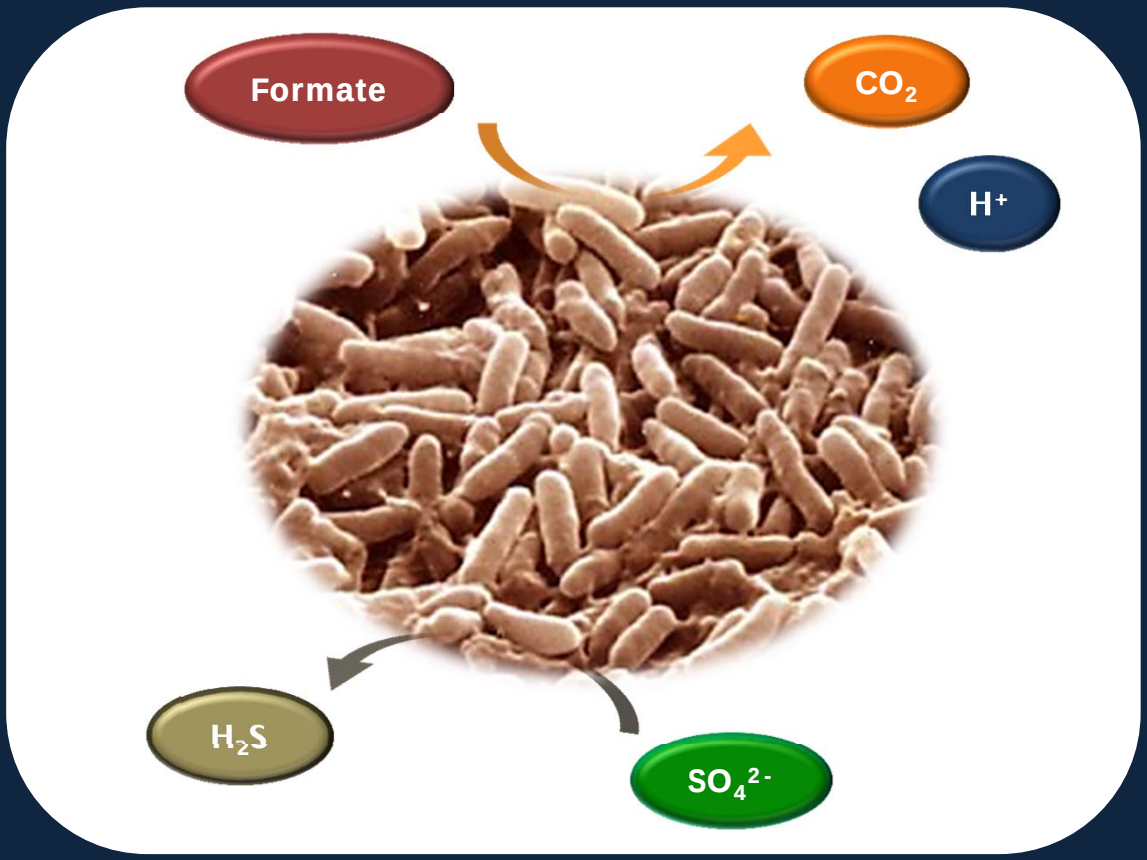
Formate Metabolism in Sulfate Reducing Bacteria

Sofia Isabel Marques da Silva



Dissertation presented to obtain the Ph.D degree in Biochemistry

Instituto de Tecnologia Química e Biológica | Universidade Nova de Lisboa

Oeiras,
September, 2011

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Cover image: Formate oxidation and sulfate reduction by sulfate reducing bacteria.

The photo shows *Desulfovibrio vulgaris*, the model organism of SRB. Source: http://failure-analysis.info/2010/06/biological-corrosion-of-metals/

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From left to right: Carlos Romão, Maria João Romão, Inês Cardoso Pereira, Sofia da Silva, Christiane Dahl, Alfons Stams

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Jury

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Foreword

The work described in this thesis was performed under supervision of Dr. Inês Cardoso Pereira in the Bacterial Energy Metabolism Laboratory at Instituto de Tecnologia Química e Biológica of Universidade Nova de Lisboa.

This thesis is divided in 8 chapters. In the first chapter a general introduction to several aspects of SRB, related to the research carried out, will be presented, followed by an introduction to the metalloproteins that were investigated in this work. This first Chapter has a general character and is complemented by the more specific information presented in the introduction to each of the other Chapters. Chapter 1 starts with an historical and evolutive perspective of SRB and biological sulfate reduction, including a phylogenetic perspective of sulfate reducing organisms, followed by a description of the main characteristics of the *Desulfovibrio* genus, and two of its most studied species, *Desulfovibrio vulgaris* and *Desulfovibrio desulfuricans*, which were the microorganisms studied in this thesis. In the following sections several aspects concerning SRB energy metabolism will be discussed, with emphasis on formate, lactate, pyruvate and hydrogen metabolism, as well as sulfate reduction. Subsequently, the metalloenzymes formate dehydrogenases and hydrogenases and their soluble electron carriers *c*-type cytochromes, will be presented. Finally, the involvement of

SRB in syntrophic associations will be discussed, highlighting the role of formate in interspecies metabolite transfer. The role of SRB in the environment and health will also be discussed, followed by an overview about the human pathogen *B. wadsworthia*.

Chapters 2 to 6 consist of original research, of which the work in chapters 2, 5 and 6 has already been published. The bioinformatic work described on Chapter 5 is part of a broader analysis of energy metabolism genes in sulfate reducing organisms, and supplementary information regarding this work can be found on Chapter 8. Concluding remarks and future perspectives are presented on Chapter 7.

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Thesis publications

Sofia M. da Silva, Sofia Venceslau, Cláudia V. Fernandes, Filipa M. A Valente, Inês A.C. Pereira (2008). *Hydrogen metabolism in the human pathogen Bilophila wadsworthia*. Antonie Van Leeuwenhoek 93, 381-390.

Sofia M. da Silva, Catarina Pimentel, Filipa M. A. Valente, Claudina Rodrigues-Pousada, Inês A. C. Pereira (2011). *Tungsten and molybdenum regulation of formate dehydrogenase expression in Desulfovibrio vulgaris Hildenborough*. Journal of Bacteriology 193, 2909-2916.

Inês A. C. Pereira, Ana Raquel Ramos, Fabian Grein, Marta Coimbra Marques, **Sofia M. da Silva**, Sofia Santos Venceslau (2011). *A comparative genomic analysis of energy metabolism in sulfate reducing bacteria and archaea*. Frontiers in Microbial Physiology and Metabolism 2 (69).

Sofia M. da Silva, Joahanna K. Voordouw, Cristina Leitão, Gerrit Voordouw, Inês A. C. Pereira (2011). *Function of formate dehydrogenases in Desulfovibrio vulgaris Hildenborough energy metabolism*. In preparation.

Sofia M. da Silva, Inês A. C. Pereira (2011). *Periplasmic electron transfer between formate dehydrogenase and cytochromes c in Desulfovibrio desulfuricans ATCC27774*. In preparation.

Publication not included in this thesis:

Shwana Johnston, Shiping Lin, Phoebe Lee, Sean M. Caffrey, Janine Wildschut, Johanna K. Voordouw, **Sofia M. da Silva**, Inês A. C. Pereira, Gerrit Voordouw (2009). *A genomic island of the sulfate-reducing bacterium Desulfovibrio vulgaris Hildenborough promotes survival under stress conditions while decreasing the efficiency of anaerobic growth*. Environmental Microbiology 11 (4), 981-991.

Dissertation Abstract

Sulfate reduction is a very ancient metabolic process and it is responsible for more than 50% of carbon mineralization in anaerobic marine sediments. Sulfate-reducing organisms (SRO) are able to couple the reduction of sulfate to the oxidation of organic compounds, such as lactate or formate, or molecular hydrogen (H_2), in order to obtain energy for cell synthesis and growth. Despite recent significant advances, a lot remains to be known about the mechanisms for energy conservation in SRO, and the specific components involved in those mechanisms. Formate and hydrogen are two abundant metabolites in SRO habitats, usually formed as fermentation products of other organisms. However, while the role of hydrogen and hydrogenases in anaerobic metabolism has been intensively studied over the years, formate has not received the same attention as an equally important metabolite. That situation has changed recently though, and formate has been increasingly recognized as a key metabolite in several processes. The importance of hydrogen in sulfate-reducing bacteria (SRB) metabolism is well patent in the fact that most of these organisms possess multiple hydrogenases. One of the enzymes implicated in formate metabolism is formate dehydrogenase (FDH), which is also present in multiple numbers in most SRB, also suggesting an important role in their metabolism.

The aim of the work presented in this thesis was to provide insight into SRB metabolic pathways, namely in formate metabolism, by studying the key enzymes, formate dehydrogenases. The role and expression regulation of FDH was addressed in *Desulfovibrio vulgaris*, a model organism of SRB, whose genome codes for three FDHs. The possible energy metabolism pathways were further examined by testing electron transfer between FDH and [NiFe]-hydrogenase and several *c*-type cytochromes from *Desulfovibrio desulfuricans*. In order to have a broader perspective on the potential energy conserving pathways in different sulfate reducers, a comparative analysis of periplasmic FDH, hydrogenase and *c*-type cytochrome genes was performed in 25 available genomes of SRO.

The study of the energy metabolism of *Bilophila wadsworthia*, a recognized opportunistic pathogen phylogenetically related to SRB, was also undertaken in this thesis.

Molybdenum (Mo) and tungsten (W) are two elements with very similar properties. Formate dehydrogenase was the first enzyme to be shown to naturally incorporate W, when this element was considered to be mostly an antagonist to Mo. Since then many tungstoenzymes have been isolated and characterized, mainly, but not exclusively, from archaeal organisms. Some molybdoenzymes were shown to be able to also incorporate W and retain their activity, while others become completely inactive. Formate dehydrogenases containing Mo or W in their active site have been reported in SRB. In the present work we showed for the first time direct evidence of

transcriptional or posttranscriptional regulation of FDH isoenzymes by Mo and W in *D. vulgaris*. The replacement of Mo by W had a profound effect in FDH activity when formate or H_2 were the electron donors. Surprisingly, the effect of W was even more pronounced in the presence of H_2 than formate. We showed by activity stained gels, Western-blot analysis, real-time qPCR and protein isolation that different isoenzymes are expressed during growth with either Mo or W, indicating the presence of a metal-dependent regulatory mechanism for *D. vulgaris* FDHs. For $FdhABC_3$, an enzyme containing a cytochrome *c*-like subunit, a high selectivity for Mo incorporation is observed, while for FdhAB, an heterodimeric enzyme, the process of metal incorporation is not so specific or tightly regulated since both metals can be incorporated.

In order to further study the role of the two main FDHs of *D. vulgaris*, mutants for each soluble FDH were generated and the effect of both mutations in growth with different electron donors was addressed. Both soluble formate dehydrogenases are important for growth on formate in the presence of Mo, whereas in W only the FdhAB plays a role, due to the repression of $fdhABC_3$. Both *ΔfdhAB* and $\Delta fdhABC_3$ display defects in growth with lactate/sulfate providing evidence for the involvement of formate cycling in this process. In contrast, both mutants grew similarly to the wild-type in hydrogen/sulfate. In the absence of sulfate, the *D. vulgaris* cells produced formate when supplied with H_2/CO_2, which resulted from CO_2 reduction by the periplasmic enzymes. We propose that this

process may be environmentally significant by allowing growth of sulfate reducing bacteria by hydrogen oxidation coupled to CO_2 reduction in syntrophy with organisms that consume formate and are less efficient in H_2 utilization.

The electron transfer pathways involving $FdhABC_3$ from *D. desulfuricans* and several *c*-type cytochromes were also investigated. $FdhABC_3$ was shown to transfer electrons efficiently to every cytochrome tested, although the higher rates were obtained for the monoheme cytochrome c_{553}, previously reported as its physiological partner. $FdhABC_3$ was also able to reduce with high rates the Type I cytochrome c_3 (TpIc_3) and the split-Soret cytochrome. Furthermore, $FdhABC_3$ was able to transfer electrons directly to each of these proteins and the presence of catalytic amounts of c_{553} or TpIc_3 did not have a significant influence in the reduction rates. $FdhABC_3$ is also able to slowly reduce NhcA, a cytochrome associated with a membrane redox complex, and more slowly Dsr, a membrane complex containing a *c*-type cytochrome which does not belong to the c_3 family.

The comparative genomic analysis revealed the presence of multiple FDHs in most SRO. All organisms, with exception of two archaea and a member of Clostridia, contain at least one periplasmic FDH. Concerning c-type cytochromes, there are two distinct groups: one composed by Deltaproteobacteria and *Thermodesulfovibrio yellowstonii*, which are characterized by a large number of multiheme cytochromes *c*, and a second group composed by members of

Archaea and Clostridia where cytochromes *c* are present in very few numbers or totally absent. The cytochrome *c*-containing group also have a higher number of periplasmic hydrogenases and formate dehydrogenases, and membrane-associated redox complexes suggesting a higher flexibility in energetic pathways.

Bilophila wadsworthia is an important opportunistic pathogen isolated from several anaerobic infections. It is a most interesting bacterium as it belongs to the *Desulfovibrionaceae* family, although is not able to reduce sulfate. This organism performs an interesting type of respiration in which taurine serves as a source of sulfite, the final electron acceptor, for the oxidation of short-chain fatty acids, such as lactate or formate, produced by fermentative organisms present in the human gut. We showed that H_2 is also used as energy source in a very efficient way by *B. wadsworthia*, which expresses up to five hydrogenases in the presence of different electron donors. H_2 is a very important source of energy for bacteria in the human gut and the ability to use it efficiently most likely constitutes a virulence factor for *B. wadsworthia*.

Resumo da dissertação

A redução de sulfato é um processo metabólico muito antigo e é responsável por mais de 50% da mineralização de carbono nos sedimentos marinhos anaeróbios. Os organismos redutores de sulfato (ORS) têm a capacidade de acoplar a redução de sulfato à oxidação de compostos orgânicos, tais como o lactato ou o formato, ou ainda ao hidrogénio molecular, de modo a obterem energia para a síntese celular e crescimento. Não obstante os mais recentes progressos, os mecanismos que permitem a conservação de energia nos ORS continuam essencialmente desconhecidos. O formato e o hidrogénio são dois metabolitos abundantes nos habitats dos ORS, geralmente formados como produtos de fermentação por outros organismos. No entanto, enquanto o papel do hidrogénio e das hidrogenases no metabolismo anaeróbio tem sido estudado intensivamente ao longo dos anos, o formato, por outro lado, não tem sido alvo da mesma atenção enquanto metabolito igualmente importante no metabolismo anaeróbio. Todavia, a situação tem vindo a alterar-se, e o formato tem vindo cada vez mais a ser reconhecido como um metabolito fundamental em vários processos biológicos. A importância do hidrogénio para as bactérias redutoras de sulfato (BRS) está bem patente no facto da maioria destes microrganismos possuir múltiplas hidrogenases. Uma das enzimas envolvidas no metabolismo do formato é a formato desidrogenase

(FDH), que por sua vez também está presente em múltiplas cópias na maioria das BRS, indicando assim que desempenham também um papel importante no seu metabolismo.

O principal objectivo do trabalho apresentado nesta tese foi contribuir para a clarificação das vias metabólicas das BRS, principalmente do metabolismo do formato, através do estudo das formato desidrogenases, enzimas fundamentais neste processo. A função e a regulação da expressão das FDHs foi estudada em *Desulfovibrio vulgaris*, um organismo modelo das BRS, cujo genoma codifica para três FDHs. As potenciais vias do metabolismo energético foram ainda estudadas através de experiências em que se testou a transferência electrónica entre a FDH ou a hidrogenase de [NiFe] e vários citocromos *c* de *D. desulfuricans*. De modo a perceber quais as potenciais vias de conservação de energia que envolvem as desidrogenases periplásmicas em diferentes ORS, foi feita uma análise comparativa dos genes correspondentes às FDHs, hidrogenases e citocromos *c* periplásmicos, em 25 genomas de ORS. Nesta tese foi ainda estudado o metabolismo energético de *Bilophila wadsworthia*, um reconhecido patogénio oportunista filogeneticamente relacionado com as BRS.

O molibdénio (Mo) e o tungsténio (W) são dois elementos com propriedades muito semelhantes. A formato desidrogenase foi a primeira enzima em que se mostrou a presença de W, numa altura em que este metal era apenas considerado um antagonista do Mo. Desde essa descoberta, muitas enzimas contendo W foram isoladas e

caracterizadas, principalmente, mas não só, a partir de organismos pertencentes ao domínio Archaea. Algumas enzimas de Mo têm a capacidade de incorporar W e manter a sua actividade, enquanto outras, ao substituir o Mo por W, são inactivadas. Formato desidrogenases contendo Mo ou W no seu centro activo têm sido reportadas em BRS. No trabalho apresentado nesta tese mostrámos pela primeira vez evidência directa de regulação transcricional ou pós-transcricional pelo Mo e W nas três FDHs de *D. vulgaris*. A substituição de Mo por W no meio de cultura tem um efeito bastante pronunciado na actividade da FDH, sobretudo quando o formato ou o hidrogénio são usados como dadores de electrões. Surpreendentemente, o efeito do W é ainda mais pronunciado na presença do H_2 do que do formato. Neste trabalho, usámos géis de actividade, Western-blot, PCR em tempo real e isolamento das proteínas, para mostrar que diferentes isoenzimas são expressas durante o crescimento com Mo ou W, sugerindo a existência de um mecanismo de regulação dependente dos metais para a expressão das FDHs de *D. vulgaris*. Observou-se que para a $FdhABC_3$, uma enzima que contém uma subunidade citocromo *c*, existe uma elevada selectividade para a incorporação de Mo, enquanto que para a FdhAB, uma enzima heterodimérica, o processo de incorporação do metal não é tão estritamente regulado, uma vez que tanto o Mo como o W podem ser incorporados.

Com o objectivo de esclarecer a função das duas principais FDHs de *D. vulgaris* foram criados mutantes para cada uma das FDHs

solúveis e os efeitos das mutações no crescimento das bactérias com diversos dadores de electrões foram analisados. As duas formato desidrogenases solúveis são importantes para o crescimento em formato na presença de Mo, enquanto que com W apenas a FdhAB desempenha uma função, devido à repressão do gene *fdhABC*$_3$. Os dois mutantes, *ΔfdhAB* e *ΔfdhABC*$_3$, apresentam deficiências no crescimento em lactato/sulfato, o que constitui uma evidência do envolvimento do 'ciclo do formato' neste processo. Por outro lado, os dois mutantes cresceram de forma semelhante ao tipo selvagem em hidrogénio/sulfato. Na ausência de sulfato, e na presença de H_2/CO_2, as células de *D. vulgaris* produziram formato que resultou da redução de CO_2 pelas enzimas periplásmicas. Nós propomos que este processo pode ser ambientalmente significativo, pois permite o crescimento das bactérias redutoras de sulfato, através da oxidação do hidrogénio acoplada à redução de CO_2, em sintrofia com organismos que consomem formato e são menos eficientes na utilização do H_2.

As vias de transferência electrónica envolvendo a $FdhABC_3$ de *D. desulfuricans* e vários citocromos *c* foram também estudadas. Os resultados mostram que a $FdhABC_3$ transfere electrões de forma eficaz para todos os citocromos testados, embora as taxas de redução mais elevadas tenham sido obtidas com o citocromo monohémico *c*$_{553}$, previamente designado como o parceiro fisiológico da FDH. As taxas de redução do citocromo *c*$_3$ do tipo I (TpI*c*$_3$) e do split-Soret são também elevadas. Além disto, verificou-se que a

$FdhABC_3$ é capaz de transferir electrões directamente para cada proteína testada e a presença de quantidades catalíticas de c_{553} ou Tplc_3 não mostraram ter uma influência significativa nas taxas de redução. Neste trabalho mostrámos também que a $FdhABC_3$ tem a capacidade para reduzir lentamente o NhcA, um citocromo associado a um complexo redox membranar, e ainda mais lentamente o Dsr, um complexo membranar que contém um citocromo *c* que não pertence à família do c_3.

A análise genómica permitiu confirmar que a presença de múltiplas FDHs é comum na maioria das BRS, e todos os organismos, com excepção de duas archaea e um membro dos Clostridia, contém pelo menos uma FDH periplásmica. Em relação aos citocromos *c*, podemos reconhecer dois grupos distintos, um composto pelas Deltaproteobacteria e pelo *Thermodesulfovibrio yellowstonii*, que se caracteriza pela presença de um elevado número de citocromos *c* multihémicos, e um segundo composto por membros das Archaea e Clostridia, que possuem muito poucos ou nenhuns citocromos *c*. O grupo dos organismos que contém citocromos c também possui um maior número de hidrogenases e formato desidrogenases periplásmicas, assim como complexos redox membranares, o que sugere uma maior flexibilidade das vias energéticas.

A *B. wadsworthia* é um importante patogénio oportunista isolado a partir de várias infecções anaeróbias. É uma bactéria singular porque, embora pertença à família *Desulfovibrionaceae*, não tem a capacidade para reduzir sulfato. Este microrganismo realiza um

tipo de respiração muito interessante em que a taurina serve como fonte de sulfito, o aceitador final de electrões, para a oxidação de ácidos gordos de cadeia curta, como o lactato ou o formato, produzidos por microrganismos fermentativos que habitam o intestino humano. Neste trabalho mostrámos que o H_2 é também usado como fonte de energia de modo muito eficiente pela *B. wadsworthia*, que expressa até cerca de cinco hidrogenases na presença de diferentes dadores de electrões. O H_2 é uma fonte de energia muito importante para as bactérias que compõem a flora intestinal humana e a capacidade para o usar eficientemente constitui provavelmente um factor de virulência para a *B. wadsworthia*.

Abbreviations

ADH	Alcohol dehydrogenase
ANME	Anaerobic methanotrophic archaea
AOM	Anaerobic oxidation of methane
AOR	Aldehyde ferredoxin oxidoreductase
APS	Adenosine phosphosulfate
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BCIP	5-bromo-4-chloro-3-indolyl phosphate toluidine salt
C_{553}	Monoheme cytochrome
ca.	circa, approximately
CoA	Coenzyme A
Cys	Cysteine
$\Delta G^{o\prime}$	Gibbs free energy change under physiological standard conditions
DMB	3,3'-dimethoxybenzidene dihydrochloride
DMSO	Dimethylsulfoxide
cDNA	Complementary deoxyribonucleic acid
Dsr	Dissimilatory sulfite reductase
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
e.g.	exempli gratia, for example
ETC	Electron transport chain
FDH	Formate dehydrogenase
Fd	Ferredoxin
FHL	Formate hydrogen lyase
FMDH	Formylmethanofuran dehydrogenase
HmcA	Cytochrome subunit from Hmc (High molecular mass cytochrome *c*) complex
HPLC	High performance liquid chromatography
IBD	Inflammatory bowel diseases
ICP-MS	Inductively coupled plasma mass spectrometry
i.e.	id est, that is
IMG	Integrated Microbial Genomes
MCD	Molybdopterin cytosine dinucleotide
MGD	Molybdopterine guanosine dinucleotide
Moco	Molybdenum cofactor
MTC	Maximum tolerable concentration
NADH	Nicotinamide adenine dinucleotide, reduced form
NAD(P)	Nicotinamide adenine dinucleotide (phosphate), oxidized form
NBT	Nitro blue tetrazolium salt
NhcA	Cytochrome subunit from Nhc (Nine heme cytochrome *c*) complex

NrfD	D subunit from Nrf (Nitrite reduction from formate) complex
OD	Optical density
PFL	Pyruvate formate lyase
PFOR	Pyruvate ferredoxin oxidoreductase
pI	Isoelectric point
Pi	Inorganic phosphate
PPi	Inorganic pyrophosphate
PMSF	phenylmethanesulfonil fluoride
PVDF	Polyvinylidene fluoride
qRT-PCR	Quantitative real time polymerase chain reaction
rRNA	Ribossomic ribonucleic acid
SCFA	Short chain fatty acid
Sec	Selenocysteine
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SLP	Substrate level phosphorylation
sp. / spp.	Species (singular / plural)
SRB	Sulfate reducing bacteria
SRO	Sulfate reducing organisms
Tat	Twin arginine translocation pathway
Tc	Technetium
TCA	Trichloroacetic acid
TMAO	Trimethylamine N-oxide
TpIc_3	Type I cytochrome c3
TpIIc_3	Type II cytochrome c3
Tuco	Tungsten cofactor
UV	Ultraviolet
WP	Widdel-Pfennig
wt	Wild-type

Units

ºC	Degree Celsius
g	Gram
h	Hour
KDa	Kilodalton
l	litter
mg	Milligram
min	Minute
ml	Milliliter
mM	Millimolar
mol	Mole
µM	Micromolar
nM	Nanomolar
nmol	Nanomole
pM	Picomolar
U	Unit

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Chapter 1

Sulfate Reducing Bacteria

1.1. An overview

The first sulfate-reducing organism (SRO) named *Spirillum desulfuricans* was isolated more than a hundred years ago by Martinus Beijerinck from a Dutch city canal in Delft [1]. Sulfide had been previously acknowledged as a product of biological sulfate reduction in aquatic habitats, but Beijerinck was able to enrich a culture and obtain isolated colonies in agar whose distinctive characteristic was the formation of a black iron precipitate in their surroundings. By adding aerobic bacteria to the growth medium, which consume the oxygen available, he was able to obtain reproducible growth for the SRO, disseminating the idea that these bacteria were strict anaerobes. Since Beijerinck time to our days many discoveries have been made about this important group of microorganisms and their ecological, physiological and metabolic diversity [2-4]. The development of genomic, biochemical, genetic and biogeochemical tools provided new insight into the diversity of SRO, allowing the isolation of new members from previously unknown habitats and revealing previously unknown energetic strategies [4].

Sulfate reduction is a very ancient anaerobic metabolism, whose first evidence dates back to 2.7 billion years ago, in the Archean. About this time low concentrations of atmospheric O_2 from oxygenic photosynthesis caused the appearance of oxidized species like iron oxides or soluble sulfate. The latter may have accumulated

in certain zones, creating restricted sulfate-rich environments that promoted biological sulfate reduction. However, only in Proterozoic, 2.3 billion years ago, high amounts of sulfide started accumulating driven by an increasing sulfate concentration, and consequent higher rate of biological sulfate reduction, establishing a sulfidic ocean until about 0.5 billion years, when a major oxidation event in the Earth's atmosphere lead to deep-water oxygenation [5-8].

SRO have been found in a wide diversity of anaerobic habitats from marine and freshwater sediments to the gastrointestinal tract of animals, including humans [9-12]. They are particularly abundant in marine sediments were sulfate concentration is high (28mM), and they play a major role in linking the sulfur and carbon cycles by their ability to use sulfate in a dissimilatory way. More than 50% of the organic matter mineralization in marine sediments is due to sulfate reduction [13-15]. SRO are able to couple the reduction of sulfate to the oxidation of organic compounds or molecular hydrogen in order to obtain energy for cell synthesis and growth [4]. Sulfate reduction metabolism results in the production of high amounts of sulfide, which serves as electron donor to a variety of aerobic or anaerobic chemotrophic and anoxygenic phototrophic microorganisms that oxidize it to elemental sulfur and sulfate, creating a cycle of sulfur species transformations [16, 17]. In man-made environments where SRO also thrive, accumulation of sulfide may constitute a problem due to the toxic and corrosive nature of this compound. Biocorrosion of concrete pipes, conduits, metal structures and souring of oil fields

poses a significant threat to many industries, mainly oil industry [18]. Sulfide, produced by sulfate-reducing bacteria (SRB) present in the human larg intestine, has also been implicated in inflammatory bowel diseases because of its toxic effects to epithelial colonic cells [19]. Nevertheless, SRO are drawing increased attention because of their potential role in bioremediation, due to their ability to grow with diverse pollutant compounds [2]. Given the economical, health and environmental importance of SRO it is essential to further understand their metabolism in order to control their undesired activity or to take advantage of all their potential.

1.2. Taxonomy

The group of SRO is heterogenous since it gathers members from different phylogenetic lineages and with a wide metabolic diversity, which have in common the ability to use sulfate as terminal electron acceptor. As mentioned before sulfate reduction is a very ancient metabolism, and several events of lateral gene transfer can help explain the dispersion of this ability by phylogenetic unrelated microorganisms [20]. The sets of genes coding for two key enzymes of sulfate reduction, adenosine phosphosulfate (APS) reductase and dissimilatory sulfite reductase (Dsr), were probably laterally transferred among different lineages in several moments of SRO evolution [21, 22].

Comparative analysis based on 16S ribosomal RNA (16S rRNA) yields seven phylogenetic lineages in SRO: Deltaproteobacteria (families Desulfovibrionaceae, Desulfomicrobiaceae, Desulfobacteriaceae, Desulfohalobiaciae, Desulfobulbaceae and Syntrophobactereacea), Nitrospirae (genus *Thermodesulfovibrio*), Thermodesulfobacteria (genus *Thermodesulfobacterium*), Clostridia (genera *Desulfotomaculum*, *Desulfosporosinus* and *Desulfosporomusa*), Thermodesulfobiaceae (genus *Thermodesulfobium*) and Archaea (genus *Archaeoglobus* from Euryarchaeota; genera *Caldivirga* and *Thermocladium* from Crenarchaeota). SRO belonging to Deltaproteobacteria are Gram-negative, usually mesophilic bacteria while Clostridia comprise Gram-positive, spore-forming bacteria. Genera *Thermodesulfovibrio*, *Thermodesulfobacterium* and *Thermodesulfobium* include only thermophilic bacteria [3, 23].

Several genomes of SRO have been recently sequenced, mostly from Deltaproteobacteria [24-27]. In fact, most of SRO described until now, belong to this class.

1.2.1. *Desulfovibrio* spp.

Research on SRO metabolism has been carried out mostly with the genus *Desulfovibrio*, since these organisms are easily manipulated in laboratory conditions. *Desulfovibrio* spp. are mesophilic Gram-negative Deltaproteobacteria, belonging to the Desulfovibrionaceae family, polarly flagellated, curved rods with no

ability to form spores and which are characterized by the presence of desulfoviridin, a type of dissimilatory sulfite-reductase. *Desulfovibrio* spp. use organic acids, alcohols or molecular hydrogen as substrates for growth with reduction of sulfate, and are incomplete oxidizers since they are not able to oxidize acetate. It is the only genus among the SRO that can be found in the digestive tract of animals and humans, and two species, *D. piger* and *D. fairfieldensis*, have actually never been isolated from habitats outside the human body [28-30]. *Desulfovibrio* spp. have been considered as strict anaerobes, but they are often found in temporary oxic zones possessing the necessary enzymes to cope with oxygen toxicity. It was recently shown that a strain is able to grow at atmospheric oxygen levels and it was previously documented that in *D. vulgaris* and *D. desulfuricans* oxygen reduction can be coupled with proton translocation and energy conservation [31-34].

D. vulgaris Hildenborough is a model organism for SRO. It is a well studied strain of *Desulfovibrio* and the first SRO to have its genome sequenced [35]. Its genome codes for several periplasmic enzymes like hydrogenases, one of the most studied enzymes, and also several formate dehydrogenases (FDH), which have received less attention. Given the number of FDHs, formate metabolism may have a central role in energy-conserving mechanisms for *D. vulgaris*, and understanding more about the role played by each FDH would be of interest to unveil the energetic pathways in this organism.

D. desulfuricans ATCC27774 is another well studied strain of *Desulfovibrio* spp., whose genome was also recently sequenced. It is one of the few strains able to use nitrate in a dissimilatory way, besides sulfate [36, 37].

Desulfovibrio spp. are characterized by possessing a high number of *c*-type cytochromes, and several types are widespread in members of this genus, of which the most abundant and well studied is the type I cytochrome c_3 (TpIc_3) [38]. However, some *c*-type cytochromes have only been isolated from *D. desulfuricans*, making it an interesting organism to study the alternative electron transfer pathways that occur in the periplasm, linking substrate oxidizing enzymes and membrane-bound electron carriers [29, 39, 40].

1.3. Energy metabolism

Diverse SRO can oxidize more than a hundred different substrates, which include, among others, lactate, formate, ethanol, malate, sugars, amino acids, aromatic hydrocarbons or alkanes and, in addition to sulfate, they may also reduce sulfite, thiosulfate, sulfur, nitrate, iron (III), fumarate and even oxygen. Molecular hydrogen (H_2) can also be used as sole electron donor with CO_2 as the only carbon source (autotrophy), or together with acetate (chemolithoheterotrophy) [3, 4, 41]. Some SRO are complete oxidizers, being able to degrade organic matter completely to CO_2, while others degrade it incompletely to acetate. In these organisms,

acetate oxidation can be accomplished by two ways: a modified citric acid cycle or via the acetyl-coenzyme A (CoA) pathway [42]. The acetyl-CoA or Wood-Ljungdhal pathway in SRO is similar to the one described for methanogenic and acetogenic organisms, and it can function to fix CO_2 in autothrophic growth or it can be reversed in order to oxidize acetate and obtain energy [43].

The SRO respiratory chain and the components involved in energy conservation are still poorly understood, although several membrane complexes have been isolated that probably contribute to this function during sulfate respiration [44]. One striking characteristic of the SRO respiratory chain is the cytoplasmic localization of all terminal reductases, which prevents their direct involvement in the establishment of a proton gradient across the membrane [29]. Given the diversity of SRO, it is likely that several respiratory chains exist, according to the electron donor used, which makes it hard to propose one single model for electron transport, except for the last reactions of sulfate activation and reduction which will be common to all pathways [4].

Studies regarding energy conservation in SRO, and the proteins involved in the process, have been carried out mainly in *Desulfovibrio* spp. In the following sub-sections the last steps of sulfate reduction, and the electron transfer chains involving formate, H_2 and lactate as electron donors will be discussed.

1.3.1. Sulfate reduction

Sulfate (SO_4^{2-}) is transported across the membrane by symport with protons in freshwater species, or with sodium ions (Na^+) in marine species [45-47], although there is at least one exception since *Desulfomicrobium baculatum*, a freshwater sulfate reducer was shown to transport sulfate with Na^+ ions and not H^+ [48]. SRO express a constitutive electroneutral transport system with a cation:sulfate symport ratio of 2:1. However, when growing under sulfate limitation, marine and freshwater SRO express a high-accumulating and electrogenic sulfate uptake sytem with a symport ratio of 3:1 [49-51]. Because two protons leave the cell with the end product of sulfate reduction, hydrogen sulfide (H_2S), by simple diffusion, the net ATP consumption will be ⅓ATP, if a stoichiometry of $3H^+$/ATP is assumed [47]. In Na^+/sulfate symport the Na^+ gradient is generated by a Na^+/H^+ antiporter [52].

Sulfate is a very stable compound which first needs to be activated by reaction with ATP, before it can be used by the cell. This first reaction is common to dissimilatory and assimilatory sulfate reduction and is catalyzed by ATP sulfurylase in the cytoplasm, forming adenosine phosphosulfate (APS) and inorganic pyrophosphate (PPi) (Eq. 1). Because PPi formation is thermodynamically unfavourable the reaction is completed by a pyrophosphatase that hydrolyzes PPi (Eq. 2) [2, 29, 53, 54]. In a few SRO, a membrane-bound proton-pumping pyrofosfatase is present, which may be involved in energy conservation [55].

Eq.1 $ATP + SO_4^{2-} \rightarrow \mathbf{APS} + PPi \quad \Delta G^{o'} = +46\ kJ/mol$

Eq.2 $PPi + H_2O \rightarrow 2Pi \quad \Delta G^{o'} = -22KJ/mol$

APS is the first electron acceptor in the respiratory chain being reduced by APS reductase to sulfite (SO_3^{2-}), which is subsequently reduced to sulfide (S^{2-}) by dissimilatory sulfite reductase (DsrAB) [29]. APS reductase is a soluble protein extremely abundant in the cell, constituting 2 to 3% of all soluble proteins in SRO of *Desulfovibrio* genus. It was already isolated from several *Desulfovibrio* spp. and also from *Archaeoglobus fulgidus* showing high similarity in its physical and chemical properties [56-60]. A membrane complex, quinone-interacting membrane-bound oxido-reductase (Qmo) is believed to be the electron donor to APS reductase, transferring electrons from the membrane menaquinone pool to the cytoplasm [61, 62]. The last step of sulfite reduction involves the transfer of six electrons to form sulfide. It was proposed previously that this reduction did not occur directly but instead in three steps with the formation of thiosulfate and trithionate as intermediates [63, 64]. Sulfite reduction, as mentioned before, is catalyzed by sulfite reductase. There are four major types of dissimilatory sulfite reductases, distinguished by spectroscopic and molecular characteristics. Desulfoviridin, a green protein characterized by containing a iron-free siroheme (sirohydrochlorin), is the one present

in *Desulfovibrio* spp. and a few other organisms [4]. Recently, the structure of *D. vulgaris* desulfoviridin (DsrAB) was solved, showing the involvement of a third protein, DsrC, in sulfite reduction, and it was proposed a four-electron reduction, instead of six, occurs with the formation of a sulfur (S^0) intermediate that is transferred to DsrC to form a persulfide. Once DsrC is released from DsrAB the persulfide is reduced forming sulfide (S^{2-}) and DsrC oxidized, which can be again reduced by the membrane complex DsrMKJOP in a cyclic reaction (Figure 1.1) [65]. It is thought that this membrane complex transfers two electrons for sulfite reduction through DsrC, possibly contributing to proton translocation while the other four electrons come from a still unknown donor [65, 66].

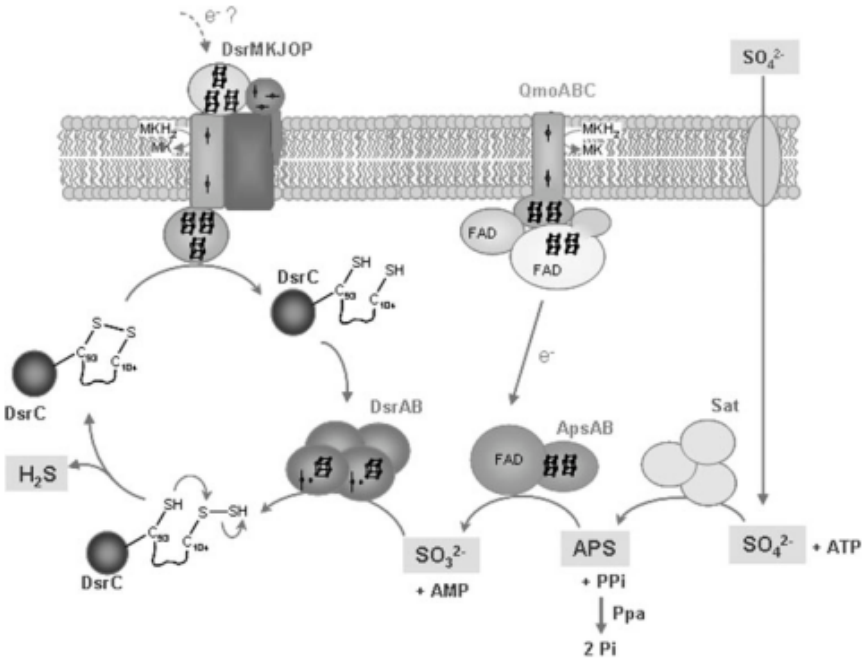

Figure 1.1. Schematic representation of the proposed sulfate reduction mechanism. Sat, sulfate adenylyltransferase; ApsAB, adenosine phosphosulfate reductase; QmoABC, membrane complex that is the probable electron donor to ApsAB. C-SH represents the thiol group of Cys-93, C-S-SH a persulfide group of Cys 104, and C-S-S-C the disulfide bond between the two Cys. From [65].

1.3.2. Formate

In anaerobic environments formate is a common growth substrate and its reversible oxidation to CO_2 is catalyzed by FDH (Eq. 3).

$$\text{Eq.3} \quad \text{Formate} \leftrightarrow CO_2 + H^+ + 2e^-$$

Formate is an important interspecies electron transfer metabolite in anaerobic mixed communities [3] where it is formed by fermentative organisms and used for growth by SRO or methanogens (see section 1.5 on Syntrophic lifestyle).

Formate can be produced by CO_2 reduction, a reaction catalyzed by FDH, or as an end product of bacterial fermentation [67]. Anaerobic facultative microorganisms, capable of glucose fermentation, such as *Escherichia coli*, produce formate as an end product in a reaction catalyzed by pyruvate-formate lyase (PFL), yielding also acetyl-CoA used for substrate-level phosphorylation (SLP) [68]. Formate is also an intermediate in the acetyl-CoA pathway for acetate oxidation to CO_2, or in the reverse way for biosynthesis of purines, aminoacids and acetate, in bacteria that use tetrahydrofolate as C1 carrier [69]. In methanogens and sulfate-reducing Archaea, however, formate is not an intermediate in C1 metabolism because these organisms lack tetrahydrofolate, although formate may be used for growth and as a source of CO_2, which is directly incorporated into formylmethanofuran [69].

Most SRO have periplasmic FDHs that oxidize formate. Energetically, as an electron donor for sulfate reduction (Eq.4), formate is equivalent to H_2 since the redox potential of the couples $2H^+/H_2$ and $HCO_3^-/HCOO^-$ is similar ($E^o \approx -0.41V$) [4].

Eq.4 $4HCO_2^- + SO_4^{2-} \rightarrow 4HCO_3^- + HS^-$ $\Delta G^{o'} = -146.6$ KJ/reaction

With formate oxidation a proton gradient is established and the resulting electrons are transferred through periplasmic electron carriers and membrane-bound complexes to reduce sulfate in the cytoplasm (for a more detailed discussion on the periplasmic electron transfers in SRO involving FDH see Chapter 5 of this thesis). Because formate oxidation and sulfate reduction occur in different sides of the membrane, energy is conserved through this process. Formate is also an intermediate in the acetyl-CoA pathway for SRB that oxidize acetate to CO_2 or that grow autotrophically with CO_2 as carbon source (e.g. *Desulfotomaculum acetoxidans* and *Desulfobacterium autotrophicum*) [70-72]. In *D. vulgaris,* a 'formate cycling' model was suggested, analogous to H_2 and CO cycling, as an energy conserving mechanism in face of the genome sequence that encodes a PFL and three periplasmic FDHs [35, 73, 74].Formate metabolism in SRB, mainly *D. vulgaris* will be further discussed on Chapter 3 of this thesis.

1.3.3. H_2

Hydrogen is a major energy source for SRO in their natural habitats and is also an intermediate in several of their metabolic pathways. It is used as energy source, with acetate and CO_2 as carbon source (chemolithoheterotrophy) or only with CO_2 (autotrophy). H_2 formation as a product of fermentative metabolism makes it an important interspecies electron transfer in anaerobic syntrophic communities. The reversible oxidation of H_2 is catalyzed by hydrogenases, a very intensively studied group of enzymes in SRO, mainly in *Desulfovibrio* genus [4, 29].

H_2 is also a major fermentation product in the human gut and the ability of some pathogenic bacteria, such as *Helicobacter pylori* or *Salmonella enterica* to efficiently use H_2 as an energy source constitutes a virulence factor [75, 76]. This subject is further discussed in Chapter 6, where the energy metabolism of the opportunistic pathogen *B. wadsworthia* was addressed.

SRO can obtain energy from H_2 oxidation with dissimilatory sulfate reduction (Eq. 5) by vectorial electron transport.

Eq. 5 $4H_2 + SO_4^{2-} + H^+ \rightarrow HS^- + 4H_2O \quad \Delta G^{o\prime} = -151.9$ KJ/reaction

In this mechanism the eight electrons, resulting from the oxidation of four H_2 molecules in the periplasm, are used for chemiosmotic ATP production, while the electrons are transferred to the cytoplasm for sulfate reduction. In fact, since at least one of

those protons is required for electrogenic symport of sulfate to the cytoplasm (see section 1.3.1 on Sulfate reduction), only seven remain available for ATP. Considering a ratio of 3 H^+/ATP, seven protons would then yield 2⅓ mol of ATP, from which 2ATP are required for sulfate activation, giving a net ATP gain for H_2 oxidation of ⅓ mol ATP [46]. Some growth experiments have contradicted this value giving a higher net gain of ATP for each mol of sulfate reduced [4]. This suggested the existence of an additional mechanism for proton translocation like vectorial proton transport through proton-pumping redox proteins or energy-conserving redox loops involving menaquinones. Several membrane complexes have been isolated and proposed to be involved in menaquinone cycling like Dsr and Qmo from *D. desulfuricans*, or more recently the quinone-reductase complex (Qrc) from *D. vulgaris* [61, 66, 77].

1.3.4. Lactate and pyruvate

Lactate is a metabolic product of several fermentative bacteria and it can be used as a substrate for growth and energy by most SRO in natural conditions. Pyruvate, on the other hand, is unlikely to be a major product of fermentative metabolism but is a cellular intermediate in lactate oxidation and other biosynthetic pathways, and is widely used for SRO cultivation in laboratory. These two organic acids can be oxidized completely to CO_2 by some SRO or incompletely to acetate by others (see section 1.3).

L- and D-lactate oxidation to pyruvate is catalyzed by $NAD(P)^+$-independent lactate dehydrogenases (LDH), enzymes that are membrane-associated and whose activity was shown in several *Desulfovibrio* spp. [4, 78, 79]. D-LDH was purified from *D. desulfuricans* and *Archaeoglobus fulgidus*, a hyperthermophilic SRO [80, 81]. Pyruvate is further oxidized by pyruvate:ferredoxin oxidoreductase (PFOR) to acetyl-CoA. This 'energy-rich' compound is then converted to acetate by phosphotransacetylase and acetate kinase, allowing SLP [82, 83]. Because one ATP is formed by lactate oxidation, and two molecules of lactate are oxidized by each reduced sulfate molecule, the net gain by substrate-level phosphorylation is zero, since two ATPs are needed to activate sulfate. So, in order to obtain energy from lactate oxidation with sulfate, an additional mechanism is necessary [4]. Regarding this question Odom and Peck, in 1981, proposed the hydrogen-cycling model for growth on lactate [84]. In this model, electrons generated by oxidation of lactate and pyruvate are converted to hydrogen by a cytoplasmic hydrogenase and released in the periplasmic space were hydrogen is again reoxidized with electrons being transferred to the cytoplasm, thus forming a proton gradient which is used for additional ATP synthesis. The main criticicisms to this theory are the non existence of a cytoplasmic hydrogenase in many SRO and the fact that hydrogen formation from lactate oxidation is an energetically unfavourable process [29, 79, 85]. Another model was proposed by Lupton et al. [86] where electrons generated from substrate oxidation in the

cytoplasm are transported through membrane carriers to sulfate reduction while protons are translocated to the periplasm (vectorial electron transport). In this case a cytoplasmic hydrogenase, or other enzyme, serves only to regulate electron flow and the levels of reduced electron carriers (e.g. ferredoxins), which results, as a side reaction, in the production of H_2 [86, 87]. Recently, evidence was provided in *D. vulgaris* for cycling of another reduced intermediate, CO, when the organism grows with lactate and sulfate [74]. The formation of H_2 from lactate oxidation can also be important for syntrophic associations and it can be an adaptation of *Desulfovibrio* spp. to survival in methanogenic environments with low or no sulfate. In this case, the removal of H_2 by other organisms lowers the gas partial pressure making H_2 production from lactate oxidation a thermodynamically favorable process [85] (see section 1.5 on Syntrophic lifestyle).

1.4. Metalloenzymes and electron carriers in SRO

The wide diversity of reactions catalyzed by proteins cannot be accounted only by the different reactive groups of amino acid residues, but resides also in the presence of cofactors, organic and/or inorganic groups that bind to the proteins and take part in catalysis [88]. Metal ions are the most common cofactors since almost half of known enzymes require their presence [89]. The use of specific metals by enzymes reflects not only their chemistry but also their evolutionary history. The biggest shift in metal availability happened 2.3 billion years ago with the rise of atmospheric O_2 which influenced the use of trace metals for biological activities. That use can be seen by analyzing genomes and proteomes of present organisms from each domain of life [89, 90]. Iron (Fe) was shown to be the predominant redox metal in biological systems, which is probably due to its high concentration in the ocean during the early evolution of living organisms [89]. When the ocean was still an anoxic environment, iron was found in its soluble form and available for biological use, but deep water oxygenation caused iron to precipitate lowering its concentration. On the other hand, other metals (e.g. zinc) that were scarce became more abundant and readily usable by organisms that appeared latter in the course of evolution [90, 91]. Fe and molybdenum/tungsten (Mo/W) are among the most common metals in oxidoreductases whose structure is known [89], and because they are essential for the proteins involved in formate and

hydrogen metabolism, their main features will be briefly discussed below.

Fe may be found associated with inorganic sulfur and cysteine residues forming [Fe-S] clusters, which are one of the oldest and more versatile cofactors, ubiquitously present in living organisms, and being able to perform several functions: as electron carriers between the enzymes active site and their redox partner, as catalysts and even as O_2 or Fe sensors [92]. [Fe-S] clusters can have different compositions and the low potential $[2Fe\text{-}2S]^{2+/+}$ and $[4Fe\text{-}4S]^{2+/+}$ clusters (Figure 1.2) are the most common, and present at 90% of analyzed unique FeS protein folds, while high potential clusters like the $[3Fe\text{-}4S]^{1+/0}$ are less represented [93]. Although these simple inorganic cofactors can be assembled spontaneously *in vitro*, in the cell the process is catalyzed by other proteins and rather complex, involving several possible different systems in prokaryotes and eukaryotes [94]. [Fe-S] clusters are thought to precede life itself and to be at the origin of the first chemical reactions that drove the origin of life in the primitive, anoxic ocean [95]. Besides being associated with S to from [Fe-S] clusters, Fe can also be found as part of heme, a cofactor that exists bound to several proteins with essential roles in energy-transduction processes, like respiration and

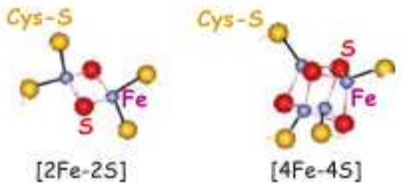

Figure 1.2. Structures of [FeS] active sites. Adapted from [93].

photosynthesis, gas transport and storage, catalysis or gene regulation. Heme is composed by a ferrous iron (Fe^{2+}) coordinated by a porphyrin macrocycle, and depending on the nature of the substituents different types of hemes can exist. The most common are hemes *b* and *c*, very similar to each other but, while heme *b* bounds non-covalently to the protein, heme *c* bounds through two covalent thioether bonds formed between cysteine side chains and the heme vinyl groups (Figure 1.3) [96]. The covalent attachment of heme may be important to confer greater stability to the protein and to allow a more dense packaging of hemes, facilitating rapid electron transfer [97]. One of the largest families of heme-containing proteins is the cytochrome *c* family. These proteins are mainly involved in electron transfer or enzyme catalysis, they may contain multiple heme groups and many times they are found assembled with other subunits forming protein complexes. Some organisms possess an incredible high number of cytochromes *c*, like *Geobacter sulfurreducens* or to a less degree, *Desulfovibrio* spp., which is thought to confer to these organisms an increased energetic flexibility [98, 99].

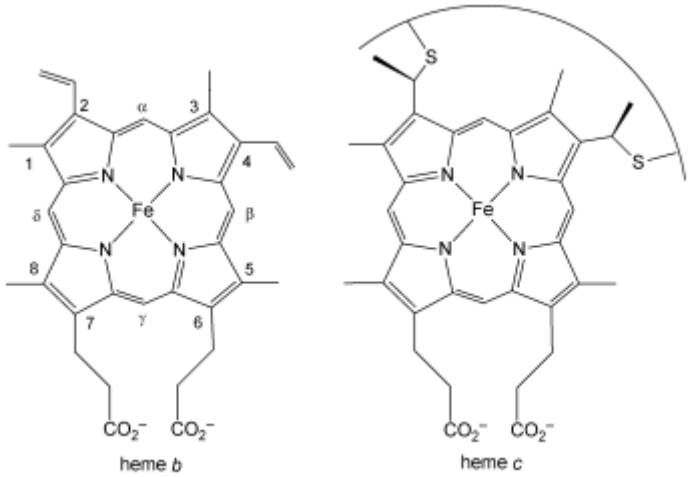

Figure 1.3. Chemical structures of heme b and heme c. The Fisher numbering system for heme substituents is shown for heme b. The curve in heme c represents a peptide segment; usually two residues separate the two Cys, and the His axial ligand follows the Cys attached to position 4. Adapted from [96].

Mo and W are transition metals that have similar chemical and physical properties but, while Mo involvement in fundamental biological processes is known for many years, W role in biology is a more recent discovery [100]. Mo is distributed among all domains of life, being an essential trace metal for microorganisms, plants and animals, while W is present only in some bacteria and archaea (Figure 1.4) [101]. The enzymes containing Mo or W in their active site have essential roles in carbon, nitrogen and sulfur metabolism [102].

The ability of Mo and W to be redox-active under physiological conditions, ranging from oxidation states VI and IV, allows the Mo or

W-containing enzymes to catalyze oxidation-reduction reactions with transfer of an oxygen atom and an exchange of two electrons at low potential [103]. However, there are exceptions, such as in the case of acetylene hydratase, a W-containing enzyme, that does not catalyze a redox reaction but instead the hydration of acetylene to acetaldehyde, or the cases of FDH and formylmethanofuran dehydrogenase (FMDH) that catalyze reactions which do not involve transfer of oxygen atoms [101, 104].

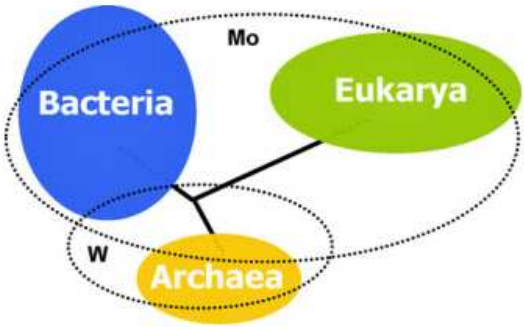

Figure 1.4. The distribution of W- and Mo-containing enzymes in the three domains of life : Mo enzymes are found in all forms of life whereas the occurrence of W enzymes appears to be restricted to archaea and some bacteria. From [101].

Mo and W are present at the active site of enzymes coordinated to an organic cofactor, pyranopterin, originating the Mo cofactor (Moco) or W cofactor (Tuco). The basic structure of pyranopterin consists of a modified pterin containing a dithiolene moiety, which quelates the metal ion with its two sulfur atoms. In eukaryotes the pyranopterin is found in the simple monophosphate form, while in prokaryotes the phosphate group is linked to a nucleotide, usually cytosine (molybdopterin cytosine dinucleotide – MCD) or guanosine (molybdopterin guanosine dinucleotide – MGD) [104]. Mo/W containing enzymes are divided in four families (Figure 1.5): 1) xanthine oxidase family, 2) sulfite oxidase family, 3)

dimethylsulfoxide (DMSO) reductase family and 4) aldehyde:ferredoxin oxidoreductase (AOR) family, according to the type of reaction catalyzed and the structure of the active site [104, 105]. From these families only the third includes molydo- and tungstoenzymes, while the enzymes belonging to the first two always contain Mo and the forth, also called the family of ''true tungstoenzymes'', includes the five AORs from *Pyrococcus furiosus* [101].

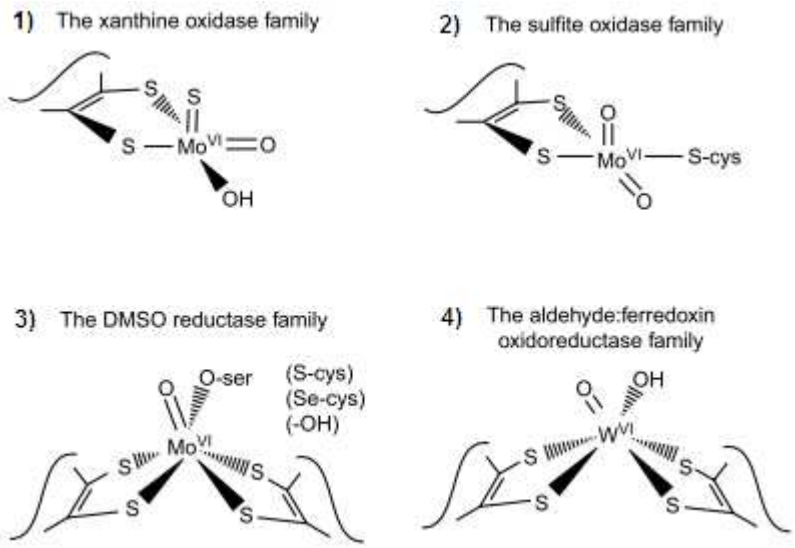

Figure 1.5. Active-site structures of Mo- and W-containing enzymes. Adapted from [105].

The fact that some enzymes incorporate only Mo, while others only W, and some incorporate both metals, probably reflects the availability of these elements in nature and their specific chemical

properties, such as the lower redox potential of the W(IV)/W(VI) couple that favors reduction of substrates instead of oxidation [100, 101]. In the primitive Earth, before the major oxygenation event that caused deep-water oxygenation, the ocean was predominantly sulfidic due to the accumulation of H_2S from biological and chemical/geological sources (see above, section 1.1). In this primordial ocean, Mo and W were present in the form of sulfides, MoS_4^{2-} and WS_4^{2-}, respectively. However, while W sulfides are relatively soluble, Mo sulfides are highly insoluble, which determined that in the primitive, anaerobic ocean W was more bioavailable than Mo [91]. As oxygen began to accumulate in the oceans, Mo and W sulfide salts were oxidized and gradually replaced by the respective oxoanions, MoO_4^{2-} and WO_4^{2-}. The very high solubility of Mo oxoanion caused a major increase in Mo availability, while the W concentration remained low, similar to the values found in the primitive ocean. The changing bioavailability of both metals has certainly conditioned protein evolution, and it is thought that W-containing enzymes are more ancient than their Mo counterparts. W is found in the active site of enzymes mainly in anaerobic bacteria and archaea, and is present at very low concentrations in marine environments. The exception occurs at anaerobic, sulfide-rich marine habitats, such as hydrothermal vents and black smokers, where W is mainly found in the form of sulfide, being more soluble, and thus more available for microorganisms that live in this environment [100, 103].

For a more detailed discussion on Mo/W and regulation of FDH expression in response to both metals see Chapter 2 of this thesis. In the following subsections a brief overview of FDH, a Mo/W enzyme, will be made, although a more detailed discussion on several aspects concerning FDH and formate metabolism can be read in Chapters 2 to 5 of this thesis. Hydrogenase and *c*-type cytochromes main characteristics will also be addressed, and more information, mainly about their involvement in periplasmic electron transfer chains, can be found in Chapters 4 and 5. The contribution of hydrogenase for *B. wadsworthia* virulence, will be discussed in Chapter 6.

1.4.1. Formate dehydrogenase

Formate dehydrogenases are metalloenzymes that catalyse the reversible conversion of formate to CO_2 (Eq.3, section 1.3.2), and are key enzymes in formate metabolism. They belong to the DMSO reductase family of Mo/W enzymes but constitute an exception in this family of oxido-reductases because they do not catalyze the transfer of an oxygen atom [106].

The importance of FDHs for microorganisms is patent in the multiplicity of compositions (number of subunits, molecular weight, metals in the active site) they can assume, the variety of electron donors/acceptors, and their catalytic efficiency, reflecting the diversity of reactions in which they can participate (Figure 1.6) [107]. FDHs can be involved in energy conservation processes through a

membrane-bound subunit that transfers the electrons from formate oxidation to other membrane-associated electron carriers, in order to reduce a final electron acceptor such as nitrate, fumarate or sulfate [107, 108]. This membrane-bound subunit is usually a cytochrome *b*, as in Fdh-N from *Escherichia coli* [109], but in *Desulfovibrio* spp. this is absent in most cases, and the electrons are transferred to *c*-type cytochromes (for a more detailed discussion see Chapter 5). FDHs can also be involved in C1 metabolism by reducing CO_2 to formate in the first step of acetyl-CoA pathway, or by oxidizing formate in the last step of acetate oxidation to CO_2 (see section 1.3) [71]. In some methanogens formate is oxidized by FDH and it can be used as a source of CO_2. Recently, the F_{420}-dependent FDH from the methanogen *Methanococcus maripaludis* was also shown to be involved in an electron bifurcation energy conserving mechanism [110, 111]. FDHs may also associate with hydrogenases in the cytoplasm, such as Fdh-H from *E. coli*, which forms a membrane-bound complex with a [NiFe]-hydrogenase [112]. In a few SRO, however, a novel putative soluble FHL was found comprising a [FeFe]-hydrogenase similar to the one described in *Treponema primitia*, but its function is, for now, unknown [55, 113].

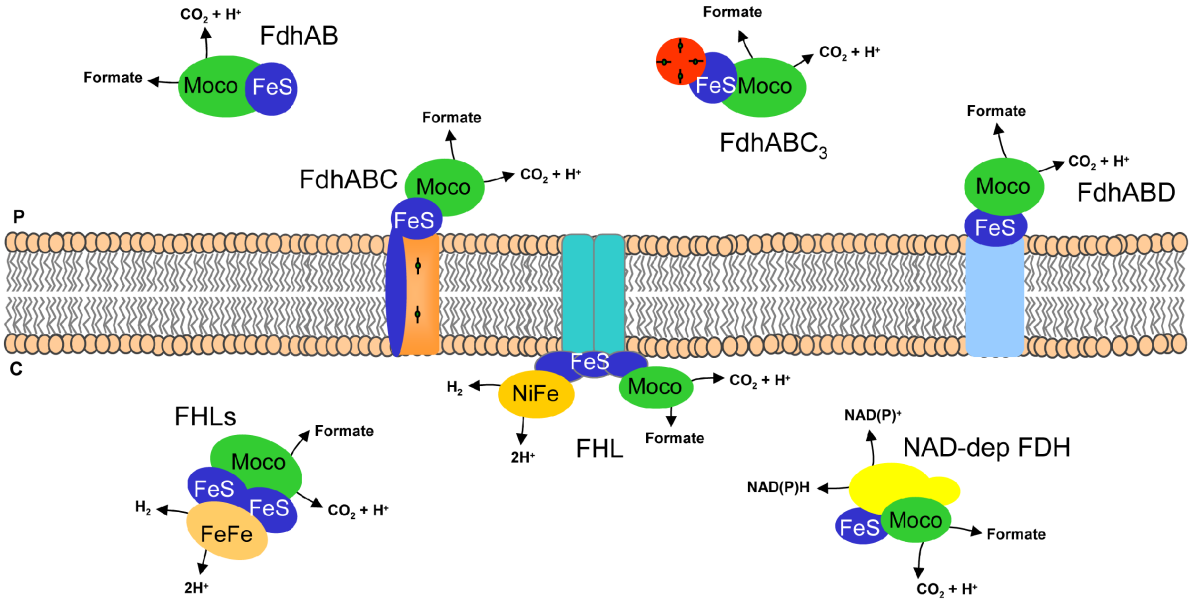

Figure 1.6. Schematic representation of several formate dehydrogenases. FdhAB, formate dehydrogenase composed by two soluble subunits. $FdhABC_3$, formate dehydrogenase containing a cytochrome *c*$_3$-like subunit. FdhABC, membrane-linked formate dehydrogenase containing a cytochrome *b* subunit. FdhABD, formate dehydrogenase associated with a membrane-linked NrfD-like subunit. NAD-dep FDH, NAD(P)-dependent formate dehydrogenase. FHL, formate hydrogen-lyase. FHLs, soluble formate hydrogen-lyase. The molybdenum (or tungsten) catalytic subunit is represented in green; subunits with iron-sulfur centers in dark blue; cytochrome c3 in red; cytochrome b in dark orange, iron hydrogenase in light orange, nickel-iron hydrogenase in dark yellow; NrfD-like subunit in light blue and NAD(P)H dehydrogenase in light yellow.

The only structure available for a FDH from a SRO, is that of *D. gigas* [114]. FDH from *D. gigas* contains W in its active site and it is composed by two subunits, where the larger catalytic subunit contains a cofactor consisting of two pyranopterins bound to guanosine (MGD co-factor) coordinating a W atom, which is also linked to a selenocysteine (Sec) from the protein side chain (Figure 1.7). The MGD cofactor is buried in the interior of the protein and is stabilized by several hydrogen bonds. This subunit contains also a [4Fe-4S] cluster and a signal peptide with the Tat motif directing its export to the periplasm, after formation of the two subunit complex in the cytoplasm. The smaller subunit contains three [4Fe-4S] clusters, but with a vacant binding site for a possible fourth cluster, by comparison to the Fdh-N from *E. coli*, where the beta-subunit has four [4Fe-4S] clusters [109,

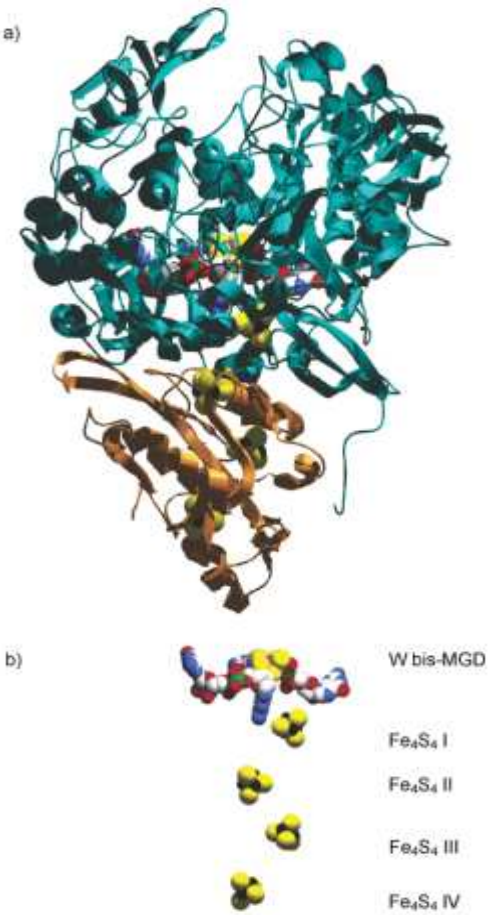

Figure 1.7. (a) Overall structure of the W-containing FDH from *D. gigas* (b) Arrangement of the redox cofactors involved in electron transfer. From [115].

114]. Other FDHs from SRO, mostly *Desulfovibrio* spp., were isolated and characterized. In *D. alaskensis* a heterodimeric FDH, similar to *D. gigas* FDH, was isolated in two isoforms, one containing Mo in the active site and the other with W [115]. In *D. desulfuricans* and *D. vulgaris* a heterotrimeric Mo-FDH was isolated containing, in addition to both subunits, a cytochrome *c*-type subunit with four hemes [116, 117]. The presence of this third subunit with *c*-type hemes is exclusive to periplasmic FDHs from SRO, and replaces the membrane-bound cytochrome *b* (see above). The multiheme subunit probably has a function in facilitating electron transfer to an acceptor partner [118]. In *Syntrophobacter fumaroxidans*, a syntrophic propionate-oxidizing bacterium also able to reduce sulfate, two W-containing FDHs (FDH-1 and FDH-2) were isolated, and shown to be extremely oxygen-sensitive contrary to what was reported for *D. gigas* FDH. FDH-2 is a heterodimeric enzyme similar to *D. gigas* FDH. Both enzymes were shown to reduce CO_2 with high activity rates [119].

All the above mentioned FDHs were shown to contain Sec coordinated to the metal in the active site. FDH is the most widespread selenoprotein and it was found in almost 90% of Sec-using organisms [120]. Selenium is present in many proteins with diverse functions, from bacteria to man [121]. It is usually present in the form of selenocysteine where it replaces sulfur in cysteine residues, and was first reported as an essential trace element for the activity of FDH in *E. coli* [122]. Se and S show differences in their reactivity that may be due to the lower pKa and higher nucleophilicity

of the selenol group versus the thiol group [123]. The presence of Sec in organisms appears to be related to environmental factors, like absence of O_2 and temperature as suggested by a genomic comparative analysis that provided evidence for the prevalence of Sec trait in anaerobic organisms living in higher temperatures [120]. SRB belonging to *Desulfovibrio* spp. are among the selenoprotein-rich organisms and *S. fumaroxidans* contains the largest selenoproteome reported to date [120].

1.4.2. Hydrogenase

Hydrogenases are metalloenzymes that catalyze the reversible oxidation of H_2 (Eq.3, section 1.3.3) and can be divided in two major classes according to the metal present in their active site: [FeFe]-hydrogenases and [NiFe]-hydrogenases. They are phylogenetically unrelated and, because they share some characteristics of the active site architecture, they represent a case of convergent evolution, where proteins with different origin converged to fulfil a similar function. A third class containing one Fe and an organic cofactor is present only in methanogens [124]. [NiFe]-hydrogenases are the most abundant and are found in Bacteria and Archaea, while [FeFe]-hydrogenases exist only in Bacteria and Eukarya [125, 126].

Regarding SRO, hydrogenases have been mainly studied in *Desulfovibrio* spp. These organisms contain both classes of enzymes and also a subgroup of [NiFe]-hydrogenases, containing a

selenocysteine coordinating the Ni in the active site [127, 128]. An early study, in which several *Desulfovibrio* spp. were screened for the genes of the three types of hydrogenases, showed that [NiFe]-hydrogenase was always present while [FeFe]- and [NiFeSe]-hydrogenases had a more limited distribution [129]. The importance of H_2 in SRO metabolism is depicted in the fact that many have multiple hydrogenases, with different cellular localizations, and different types coexist in the same organism contributing to a high metabolic flexibility [126]. Bellow, some characteristics of each type of hydrogenase are described.

[FeFe]-hydrogenases are highly modular enzymes. They share a common C-terminal domain with ca. 350 residues that is highly conserved with a unique protein fold, and which binds the H-cluster composed by a binuclear iron site bound to a [4Fe-4S] cluster by a bridging cysteine [126]. This is the minimum common domain to all [FeFe]-hydrogenases, and the smallest monomeric proteins are composed only by the H-cluster domain. Other widely distributed [FeFe]-hydrogenases have additional [4Fe-4S] N-terminal domains homologous to bacterial ferredoxin [125].

In *Desulfovibrio* spp. periplasmic [FeFe]-hydrogenases are dimeric enzymes derived from monomeric hydrogenases by splitting of the C-terminus and insertion of a signal peptide [125]. The [FeFe]-hydrogenase from *D. desulfuricans* was the first of this class to have its structure determined, which revealed that the unusual binuclear

iron site is coordinated almost exclusively by non-protein ligands, with the exception of the bridging cysteine, suggesting that the active site may have been acquired as a functional unit from the inorganic world [130]. In *D. vulgaris* it was shown, upon deletion of the [FeFe]-hydrogenase gene, that this periplasmic hydrogenase is essential for H_2 uptake [131]. However, more recently it was shown that *hydAB* (which codes for the [FeFe]-hydrogenase) was upregulated when *D. vulgaris* grew in syntrophy with a methanogenic partner, suggesting that it is involved in H_2 production from lactate oxidation [132]. A role for this hydrogenase in protection against oxidative stress has also been proposed in *D. vulgaris* [133].

The first crystal structure for a hydrogenase was obtained for [NiFe] hydrogenase of *Desulfovibrio gigas* [134]. Other [NiFe] hydrogenases were isolated from *Desulfovibrio* spp. and also from a thermophilic SRO, *Thermodesulfobacterium mobile* [135-138]. They are all periplasmic, heterodimeric enzymes, where the larger subunit carries Ni and Fe, and the smaller one carries three

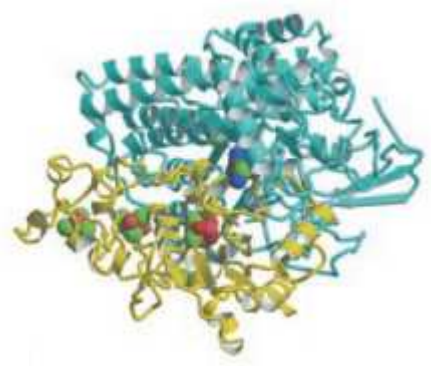

Figure 1.8. Ribbon diagram of the three-dimensional structure of [NiFe]-hydrogenase from *D. desulfuricans* showing the secondary structure elements. The small subunit is drawn in yellow, the large subunit is drawn in cyan and the prosthetic groups (including the Mg^{2+} ion and the two putative H2S molecules) are drawn in *space-filling* mode. Adapted from [137].

[Fe-S] clusters: two low potential [4Fe-4S] and the high potential [3Fe-4S] (Figure 1.8) [139].

In other organisms this type of periplasmic hydrogenase is usually membrane-bound through a membrane cytochrome *b* subunit that transfers electrons to the quinone pool and is thus involved in energy conservation [140]. However, in *Desulfovibrio* spp. all periplasmic [NiFe]-hydrogenases isolated so far lack this membrane-bound subunit [141], as above described for FDH. Cytoplasmic, multi-subunit, membrane-bound [NiFe]-hydrogenases like Ech or Coo are also found in some SRO [35, 142], although none has yet been isolated, and their exact role in energy metabolism remains to be clarified. Ech hydrogenase subunits are more related to subunits from respiratory complex I than to subunits from other [NiFe]-hydrogenases, suggesting that it may function as a proton pump, using ferredoxin as its redox partner [143].

A [NiFeSe]-hydrogenase was first isolated from a SRO, *Desulfomicrobium norvegicum*, and shown to contain equimolar amounts of Ni and Se [144, 145]. [NiFeSe]-hydrogenases of SRB are heterodimeric enzymes and their gene sequences share some degree of homology with the correspondent sequences of [NiFe]-hydrogenases and are clearly related with them [146]. It was shown for several characterized [NiFeSe]-hydrogenases that they display higher catalytic activities than [NiFe]-hydrogenases, are more tolerant to O_2 since they are isolated in a quickly reactivated state,

not requiring a long activation step as standard [NiFe] hydrogenases [147-150]. Another structural difference between the two types of hydrogenases is the replacement of the [3Fe-4S] cluster in [NiFeSe]-hydrogenase by a [4Fe-4S] [151, 152]. [NiFeSe]-hydrogenases from *D. vulgaris* and *Desulfomicrobium baculatum* can be found in a soluble or membrane-bound form in the cell. The [NiFeSe]-hydrogenase from *D. vulgaris* was shown to be a bacterial lipoprotein, that binds the membrane through a lipidic group located at the N-terminus of the large subunit, and it was the first lipoprotein shown to be translocated across the membrane by the twin-arginine translocation (Tat) pathway [153].

In *D. vulgaris* one periplasmic hydrogenase of each type was already isolated and characterized, and its genome codes for another four, two membrane-bound, cytoplasm faced, already referred, Ech and Coo, a second periplasmic $[NiFe]_2$-hydrogenase, and a cytoplasmic bifurcating [FeFe]-hydrogenase [35, 55]. Thus, this organism provides an ideal model for addressing the role of each type of hydrogenase in energy metabolism. Concerning this matter, previous work showed that Se and H_2 regulate gene expression of periplasmic hydrogenases in *D. vulgaris* [154]. Se, when added to the growth medium has a positive regulation effect in [NiFeSe]-hydrogenase, while both $[NiFe]_1$- and [FeFe]-hydrogenases are strongly downregulated. As expected, H_2 increases expression of the three hydrogenases confirming their similar function in H_2 uptake in

D. vulgaris [155]. Another work showed that [FeFe]- and [NiFeSe]-hydrogenases gene expression was also influenced by H_2 partial pressure as a reflex of the different affinities for this gas [156]. [Fe]-hydrogenase has a higher catalytic efficiency and a lower affinity being the preferred enzyme in high H_2 pressures, while [NiFeSe]-hydrogenase has a lower catalytic efficiency but a higher affinity specially indicated for scaveging lower H_2 concentrations [156]. Both studies confirm the versatility of *D. vulgaris* and the ability to adapt to changing environmental conditions, whether it is trace metal or electron donor availability.

1.4.3. Periplasmic *c*-type cytochromes

The *c*-type cytochromes have an essential role in energy transduction processes. They are widespread among prokaryotes, although the content is variable and not all organisms possess them. SRO from *Deltaproteobacteria* are usually rich in periplasmic *c*-type cytochromes, and the most abundant in *Desulfovibrio* spp. is the TpIc_3 (Figure 1.9) [29]. Several structures of this monomeric cytochrome, from different organisms, have been determined and, although they have low sequence homology, the three-dimensional fold and heme arrangement is highly conserved [38]. They have a molecular mass of about 13 KDa and contain four *c*-type heme groups with a bis-histidine coordination, and low redox potentials (-120 to -400 mV). The hemes are perpendicularly arranged among

themselves and they have a characteristic CXXCHH motif. In addition, they are characterized by a positive surface charge around heme four, that is proposed to be the site of interaction with a negative region of hydrogenase [29].

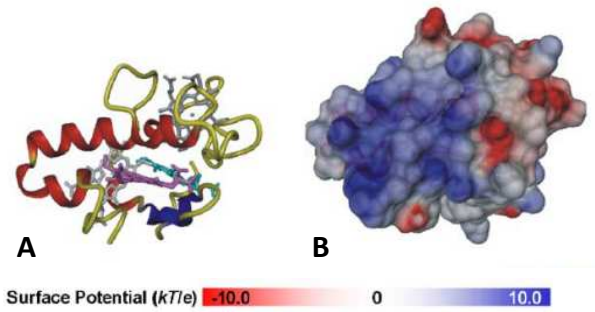

Figure 1.9. View of the fully oxidized three-dimensional structure of *D. desulfuricans* ATCC 27774 TpIc_3 cytochrome, showing its overall protein fold, secondary structure, heme arrangement and electrostatic potentials mapped at the molecular surface. **A** is a molecular diagram looking down heme 4 edge, where heme 1 is coloured cyan, heme 2 is coloured dark grey, heme 3 is coloured light grey and heme 4 is coloured violet. **B** is a view of the electrostatic potential mapped at the molecular surface in the same orientation as **A**. Adapted from [29].

A second type of cytochrome c_3 was isolated from *Desulfovibrio africanus* [157] and *D. vulgaris* [158]. Since it has different structural and reactivity properties, it was designated by TpIIc_3 cytochrome [158]. This cytochrome was first known as acidic cytochrome c_3 because in *D. africanus* it has a lower isoelectric point (pI) than TpI-c_3. However, in other organisms the TpIc_3 may also have a low pI, thus the designation concerning the pI is misleading [159]. In *D. vulgaris*

the TpIIc_3 is part of a membrane-bound complex [160]. Other *c*-type cytochromes are also part of transmembrane redox complexes like HmcA from *D. vulgaris*, containing sixteen *c*-type hemes, or 9HcA from *D. desulfuricans* [40, 161, 162]. These cytochromes, together with TpIIc_3 show a low reactivity with hydrogenase, which increases significantly when TpIc_3 is also present, suggesting that the last one mediates electron transfer between periplasmic dehydrogenases and membrane-bound complexes containing *c*-type cytochromes.

A soluble homodimeric cytochrome, named split-Soret (because of a spectroscopic characteristic), was isolated from *D. desulfuricans* [163]. It is a dimer that contains two *c*-type hemes for each subunit, parallel to each other and attached to the end of the protein being completely exposed to the solvent. It exhibits an unusual characteristic since the Fe atom in each heme from one monomer is coordinated by an histidine residue from the other monomer [39]. In addition to multiheme cytochromes, a small periplasmic monohemic cytochrome, c_{553}, is found among some *Desulfovibrio* spp. It was shown that cytochrome c_{553} is able to accept electrons from [Fe]-hydrogenase and FDH from *D.vulgaris* [118, 164, 165]. However, the fact that its gene is located near cytochrome *c*-oxidase suggests that it may have a role in protection against oxidative stress [35].

1.5. Syntrophic lifestyle

In anaerobic environments, where inorganic electron acceptors like oxygen, sulfate or nitrate are not available or are only available in very low concentrations, the complete degradation of complex organic matter to CO_2 and methane (CH_4) depends on heterogeneous microbial communities, comprising organisms with diverse metabolic abilities [166]. In these communities the association of two or more partners creates a feeding chain where the end products of one constitute the energy source of the next. This kind of cooperation, where the partners depend on each other for growth, is designated by syntrophy and always involves the transfer of one or more intermediate metabolites between the partners [167, 168]. In a syntrophic community the primary fermenters hydrolyze complex polymers such as polysaccharides, proteins or lipids, to sugars, aminoacids or fatty acids and ferment these further to short chain fatty-acids, aromatic acids, H_2, CO_2 or one-carbon compounds. The last ones, H_2, CO_2, methanol or formate, can be degraded directly to CH_4 by methanogens, but the first ones need to be further fermented by a group of secondary fermenters [166]. Many bacteria are obligate syntrophs because they are able to grow on substrates that are not easily fermentable only if the end products are removed by another partner, due to thermodynamic constrains [168]. The anaerobic organisms living in syntrophy subsist with very small amounts of energy, near to thermodynamic equilibrium, where the free energy available is close to zero ($\Delta G' \approx 0$ KJ.mol^{-1}). The minimum free energy

(Δ*G*') at which metabolism stops is variable and depends on the syntrophic partners involved as well as the steps needed for substrate activation [169].

The organisms capable of engaging in syntrophy are phylogenetic diverse and can be found in the groups of Deltaproteobacteria (genera *Syntrophobacter* and *Desulfovibrio*, among others) and the low G+C Gram-positive bacteria (genus *Syntrophomonas*, among others) [167].

In sulfate-rich environments, like marine sediments, the removal of fermentation end products can be achieved also by SRO, which are metabolically more versatile than methanogens, and can use the end products from the primary fermentations [166, 170]. SRO metabollic versatility is also reflected in the fact that in the absence of sulfate they can ferment organic acids and alcohols, producing acetate, H_2 and CO_2, whose consumption by a methanogenic partner will maintain the process viable. This ability to shift their metabolism allows SRO to adapt and thrive in environments where sulfate concentration is too low to permit respiration [171]. Several syntrophic associations between *D. vulgaris* and methanogenic archaea have been described where the bacterial partner degrades lactate, producing acetate, H_2 and CO_2 as intermediates that are consumed by the methanogen in the absence of sulfate [172-174]. In two recent studies, it was also shown that different genes in *D. vulgaris* are implicated in the different lifestyles, and different electron transfer pathways are activated when the

organism grows in syntrophic association as opposed to sulfidogenic metabolism [132, 171]. A striking aspect of some syntrophic communities is that the roles of each partner can be reversed, i.e. methanogens are also able to produce H_2, given they are in an environment with low H_2 concentration [175]. A syntrophic association between *D. vulgaris* and *Methanosarcina barkeri* was previously demonstrated where the methanogen and the bacterium grew by methanol oxidation with H_2 transfer for sulfate reduction [176]. Other syntrophic associations in sulfate reducing environments that do not involve methanogenic organisms have also been described like the one between *Syntrophus aciditrophicus* and *Desulfovibrio* sp. strain G11 (Figure 1.10), where degradation of aromatic compounds and fatty acids by the coculture depended on the presence of sulfate [177, 178].

Although H_2 is usually considered the main interspecies electron carrier in syntrophic associations, it has been increasingly recognized that formate is essential, mainly when partners do not form tight associations [168, 179, 180]. Metabolic flux calculations suggest formate may be more important than H_2 [181]. Formate can be used as the sole interspecies electron carrier as it was shown in a co-culture of *Eubacterium acidaminophilum*, an aminoacid fermenter, and an hydrogenase-negative sulfate reducer, capable of growing with formate [182]. Also, in a co-culture of *S. fumaroxidans* with methanogens, growth on propionate was only detected when the methanogenic partner was able to use both hydrogen and

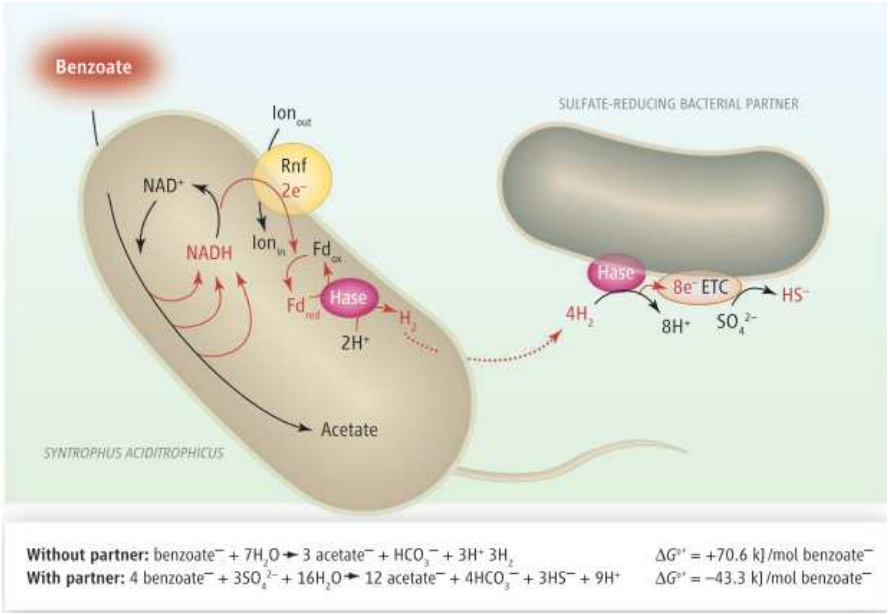

Figure 1.10. Hypothetical electron transfer between *S. aciditrophicus* and a sulfate-reducing bacterial partner during the anaerobic oxidation of benzoate. The scheme traces the interspecies transfer of electrons (red) from the source reductant (benzoate) to the terminal oxidant (sulfate). Efficient removal of hydrogen by the sulfate-reducing bacterial partner increases the amount of free energy available in the anaerobic oxidation of benzoate to acetate. Rnf, ion-translocating electron transport complex; Fd, ferredoxin; Hase, hydrogenase; ETC, electron transport chain; ΔG°', Gibbs free energy under physiological standard state conditions. From [178].

formate [183]. The high levels of FDH activity in a co-culture of *S. fumaroxidans* and *Methanospirillum hungatei* provided another evidence for formate as a preferred interspecies electron carrier [184]. Recently it was shown that formate can also be fermented by anaerobic microbial communities, allowing syntrophic growth [172]. Formate had not been regarded as a fermentable substrate since most of the methanogens can also use it directly for methanogenesis, which is an energetically more viable process. Nevertheless, other

compounds used directly by some methanogens, like acetate or ethanol, have also been shown to be substrates for syntrophic communities in certain environmental conditions [172].

Syntrophy has also been proposed as an important mechanism in microbial communities where anaerobic oxidation of methane (AOM) occurs coupled to sulfate reduction [185]. In this process methane is converted by anaerobic methanotrophic archaea (ANME), through reverse methanogenesis, to an intermediate metabolite that is consumed by autotrophic sulfate-reducing bacteria. In spite of much effort the intermediate in this process it is still unknown, although it has been suggested that multiple substrates may be involved [168, 186, 187].

1.6. Health, environmental and biotechnological impact

SRO are not only ubiquitously present in nature but also in man-made environments. Their ability to reduce sulfate has a major impact in human activities and health, due to the toxicity and corrosive nature of H_2S, the end product of sulfate reduction. However, their versatile metabolism makes them very attractive for biotechnological purposes. Several aspects concerning the ways SRO metabolism impacts our lives will be discussed below.

SRO are long known to cause biocorrosion of metal and concrete structures, like oil pipes, tanks or sewage pipes, resulting in economical losses for many industries. Corrosion is an electrochemical naturally occurring process that involves a series of oxidation (anodic) and reduction (cathodic) reactions. These reactions can be greatly influenced by microbial communities [188] that attach to the metal surface forming biofilms and allowing the establishment of an anaerobic layer close to the metal, thus promoting SRO development, and enhancing corrosion [189]. Microbial corrosion is a complex process involving several mechanisms [190]. One of the proposed mechanisms is cathodic depolarization, involving an electron transfer pathway from the ferrous metal surface to oxygen as a final electron acceptor. In this case, SRO are responsible for H_2 oxidation at the cathodic site with consequent formation of H_2S that in its turn precipitates Fe,

originating FeS corrosion products. Abiotic or biotic oxidation of FeS in the oxic zone leads to deposition of more corrosion products like Fe oxide/hydroxide [191]. Detection of hydrogenase activity from SRO was shown in corroded pipelines, and also to be maintained independently of microbial viability [192, 193]. SRO are also a concern for concrete and stonework biocorrosion. In this case H_2S resulting from their activity is converted by sulfide-oxidizing bacteria to sulfuric acid that slowly dissolves concrete [2]. Microbial activity in the oil industry has received much attention due to the importance that fossil fuels still hold as an energy source. Several biotechnologies have been proposed and/or are under development, wether to control SRO undesired activity and consequent oil souring, (e.g. by applying nitrate injections), or to take advantage of microbial fermentations thus enhancing oil recovery [18].

The ability of SRO to degrade pollutant organic compounds or heavy metals, and remove them from the environment, has drawn attention to these microorganisms as important agents in bioremediation.

The anaerobic degradation of aromatic compounds is a recent discovery, and although most of the biochemical pathways and proteins involved remain unknown, many important advances were made in this area. Usually anoxic conditions prevail in many habitats containing toxic aromatic compounds (e.g. aquifers, aquatic sediments, submerged soils or sludge digestors), thus anaerobic

microorganisms are important players in the biochemical transformations occurring in these environments [194]. Degradation of several aromatic compounds, which may be used as electron donors or acceptors, has been reported for several SRO [195-197]. Most SRO, capable of degrading these compounds, isolated until now are complete oxidizers, with the exception of *Desulfovibrio inopinatus* [198].

Benzene is a constituent of petroleum important for industry, and an environment contaminant. It is one of the least reactive aromatic hydrocarbons and needs to be activated prior degradation. Some advances have been made in understanding the mechanisms involving benzene oxidation by SRO an it was proposed an activation through conversion to benzoate [199]. Benzoate can be converted to benzoyl-CoA, which is a common intermediate in degradation pathways of other aromatic compounds such as toluene [200]. Usually, SRO involved in degradation of aromatic hydrocarbons are found in mixed communities [201], but at least two sulfate-reducing strains, capable of oxidizing naphthalene or toluene, respectively, in pure culture, were isolated [202, 203].

In littoral marine sediments contaminated with petroleum hydrocarbons, biodegradation by sulfate-reducing communities is one of the most important anaerobic processes, since sulfate is present in high concentrations. These communities are dynamic and subjected to changes over time when in presence of oil contaminants, as it was shown in a recent experiment that

characterized anaerobic hydrocarbon degradation in natural conditions [204].

Sulfide, produced by SRO as a result of their sulfate metabolism, reacts with cationic metals originating highly insoluble metal sulfides. In addition, in the absence of sulfate, many SRO can also use metals as electron acceptors. These properties make them suitable candidates for bioremediation of heavy metal contaminated wastewaters, resulting from mining activity or industrial discharges [2]. Nonetheless, immobilization of heavy metals is dependent on their concentration in solution, because they can become toxic for the bacteria, reducing their metabolic activity or even causing death. In an experiment to determine maximum tolerable concentrations (MTC) for several metals it was found that a strain of *D. vulgaris* is less tolerant then another *Desulfovibrio* sp., and copper (Cu(II)) was the most toxic metal for both cultures [205]. The toxic effects of uranium (U(VI)) for *D. desulfuricans* G20 were also determined, and shown to be dependent of the uranium species formed, as more soluble uranium complexes had no toxicity opposed to more insoluble complexes [206]. The presence of ferrous iron was shown to increase U(VI) reduction by *Desulfovibrio aerotolerans*, a SRO isolated from a U(VI) contaminated site [207].

The reduction of toxic metals can be coupled to electron transport, although not energy-conserving. The only SRO shown to be capable of growth with metal reduction is a strain of *Desulfotomaculum reducens*, isolated from heavy metal

contaminated sediments [208]. H_2 and also formate, although to a lower extent, were shown to be the preferred electron donors for technetium Tc(VII) reduction for several strains of *Desulfovibrio* spp. *D. desulfuricans* was also able to reduce tellurium (Te(IV)) and selenium (Se(VI) and Se(IV)) with excess formate [209]. Hydrogenases and TpIc_3 cytochrome were shown to be involved in the reduction mechanism of chromium (Cr(VI)), one of the most common polluting metals [210]. Other reports confirm the function of hydrogenases as chromium reductases, specially [Fe]-hydrogenase from *D. vulgaris* Hildenborough [211, 212].

The presence of SRO, belonging to *Desulfovibrio* genus, in the human large intestine was demonstrated a long time ago [213]. The human, and other animals gut comprises several microbial species that are important for maintaining mucosal equilibrium. Fermentative bacteria degrade complex carbohydrates originating several end products, like lactate, acetate or H_2, which can be metabolized by SRO and methanogens. A diet rich in sulfate may cause its accumulation in the large intestine favoring sulfate-reduction metabolism over methanogenic [214]. The consequence is the accumulation of large amounts of H_2S, a toxic compound that has been associated with inflammatory bowel diseases (IBD) like ulcerative colitis or Crohn's disease [19]. One of the most common end products of bacterial fermentation is butyrate, a short chain fatty acid (SCFA), which is an essential energetic compound for colonic

cells. However, the presence of H_2S impairs oxidation of butyrate by colonic cells, besides other toxic effects that lead to colonic epithelial inflammation [215]. Reports have shown that *Desulfovibrio* spp. are usually more prevalent in patients with inflammatory bowel diseases than in healthy persons [216, 217]. Besides being associated with IBD, *Desulfovibrio* spp. have been isolated from various infected tissues, suggesting that they may be opportunistic pathogens [30, 218]. Until now four species of *Desulfovibrio* were isolated from human samples: *D. desulfuricans*, *D. piger*, *D. fairfeldensis* and *D. vulgaris* [30, 219-221]. While *D.piger* was found to be the most abundant species, in diseased as well as in healthy persons, *D. fairfeldensis* was shown to be the most resistant to antibiotics [222]. The chemical profile of *D. desulfuricans* lipid A, a typical endotoxin in outer membrane of Gram-negative bacteria, was shown to be similar to the one of *E. coli* and *Salmonella enterica*, two pathogenic strains [223]. Recently, *Desulfovibrio* spp. have also been implicated as a possible important cause for regressive autism [224, 225].

In the next section and Chapter 6 of this thesis some aspects concerning *B. wadsworthia* are discussed. This organism is a member of *Desulfovibrionacea* and a recognized opportunistic pathogen.

1.7. A human pathogen related to *Desulfovibrio* spp.: *Bilophila wadsworthia*

Bilophila wadsworthia is a strictly anaerobic, gram-negative bacterium first isolated from patients with gangrenous and perforated appendicitis [226]. It is the third most common isolate recovered from patients with these conditions and, being an opportunistic pathogen it has also been reported in other anaerobic infections [227-229]. *B. wadsworthia* is part of the normal human fecal flora and is present in oral and vaginal fluids [228]. Isolates were also reported in other animals like pigs, chickens, felines and primates [230-232], as well as in environmental samples [233].

RNA-based phylogenetic studies showed that *B. wadsworthia* is a species closely related to *Desulfovibrio* although not able to reduce sulfate [226, 234, 235]. Instead, *B. wadsworthia* performs a different type of respiration in which taurine, an organic sulfonate, is anaerobically dissimilated to sulfide, a compound with toxic effects in human colonic cells, as mentioned above [233, 236-238]. Taurine, a metabolic product of cysteine or methionine, is one of the major organic solutes in mammals and has several important physiological functions in anti-oxidation, osmoregulation, organ development or anti-inflammation [239]. The taurine degradation pathway by *B. wadsworthia* and the intervenient enzymes are well characterized (Figure 1.11). The initial steps include a transamination reaction catalyzed by taurine:pyruvate aminotransferase that converts taurine to sulfoacetaldehyde with the consequente release of ammonia

[233, 236, 237]. The last step is the formation of sulfite (with release of acetate), which will accept electrons at the end of a respiratory chain in an energy-conserving process [233]. Sulfite is converted to sulfide by a dissimilatory sulfite reductase (Dsr) [229]. The presence of a Dsr and the phylogenetic proximity to sulfate-reducing bacteria may indicate that *Bilophila* organisms once possessed the ability to reduce sulfate, which was probably lost due to an adaptation to the human host. Not much is known yet about the beginning of the respiratory chain, how energy sources are metabolized or what proteins are involved in the electron transfer pathways. Considering the pathogenic character of *B. wadsworthia* it would be important to get further insight into its energy metabolism in order to control possible virulence factors.

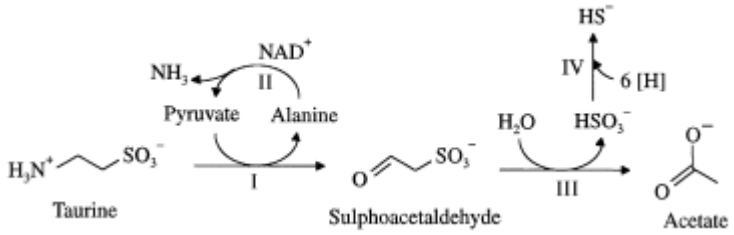

Figure 1.11. The degradative pathway of taurine in *B. wadsworthia*. Four enzymes are involved in the reduction of taurine: a taurine:pyruvate aminotransferase (I), an alanine dehydrogenase (II), a sulphoacetaldehyde sulpholyase (III) and a sulphite reductase (IV). The electrons ([H]) utilized for the reduction of sulfite derive mainly from the oxidation of an external electron donor, e.g. formate. From [238].

1.8. References

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Chapter 2

Tungsten and Molybdenum regulation of Formate dehydrogenase expression in *Desulfovibrio vulgaris* Hildenborough

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2.1. Summary

Formate is an important energy substrate for sulfate-reducing bacteria in natural environments, and both molybdenum and tungsten-containing formate dehydrogenases have been reported in these organisms. In this work we studied the effect of both metals in the levels of the three formate dehydrogenases encoded in the genome of *Desulfovibrio vulgaris* Hildenborough, with lactate, formate or hydrogen as electron donors. Using Western blot, quantitative real-time PCR, activity-stained gels and protein purification we show that a metal-dependent regulatory mechanism is present, resulting in the dimeric FdhAB protein being the main enzyme present in cells grown in the presence of tungsten, and the trimeric $FdhABC_3$ the main enzyme in cells grown in the presence of molybdenum. The putatively membrane-associated formate dehydrogenase is only detected at low levels after growth with tungsten. Purification of the three enzymes and metal analysis shows that $FdhABC_3$ specifically incorporates Mo, whereas FdhAB can incorporate both metals. The FdhAB enzyme has a much higher catalytic efficiency than the other two. Since sulfate reducers are likely to experience high sulfide concentrations that may result in low Mo bioavailability, the ability to use W is likely to constitute a selective advantage.

2.2. Introduction

Formate is a key metabolite in anaerobic habitats, arising as a metabolic product of bacterial fermentations, and functioning as a growth substrate for many microorganisms (for example methanogens and sulfate reducing bacteria). Formate is also an intermediate in the energy metabolism of several prokaryotes, and a crucial compound in many syntrophic associations, whereby organisms live close to the thermodynamic limit [1, 2]. Recent reports indicate that formate plays an even more important role in anaerobic microbial metabolism than previously considered [3-5]. The key enzyme in formate metabolism is formate dehydrogenase (FDH) [6], a member of the dimethyl sulfoxide reductase (DMSO) family. It catalyzes the reversible two electron oxidation of formate or reduction of CO_2, and plays a role in energy metabolism and carbon fixation. In anaerobic microorganisms FDH includes a molybdenum or tungsten *bis*-(pyranopterin guanidine dinucleotide) cofactor and iron-sulfur clusters [7, 8], and shows great variability in quaternary structure, physiological redox partner and cellular location [6, 9-11].

FDH was the first enzyme shown to naturally incorporate tungsten, at a time when this element was mostly considered to be an antagonist to molybdenum [12, 13]. Since then several tungstoenzymes have been isolated and characterized, mainly but not exclusively, from archaeal organisms, including FDHs, formylmethanofuran dehydrogenases (FMDH), aldehyde oxidoreductases (AOR) (not belonging to the xanthine oxidase

family), and acetylene hydratase [7, 8, 14-17]. FDHs and FMDHs can naturally incorporate either tungsten or molybdenum. Since these two elements have very similar chemical and catalytic properties, several studies have addressed the effect of substituting molybdenum for tungsten [18]. Some molybdoenzymes are able to incorporate tungsten and retain activity (*e.g. E.coli* TMAO reductase [19] or *Rhodobacter capsulatus* DMSO reductase [20]), whereas this substitution results in production of inactive enzymes in other cases such as bacterial nitrate reductases [21, 22] or *Methanobacterium formicicum* FDH [23]. In contrast, a fully active W-nitrate reductase was recently reported in the archaeon *Pyrobaculum aerophilum*, which lives in a high tungsten environment [24]. Substitution of molybdenum for tungsten has been reported for an acetylene hydratase, in which the Mo-substituted enzyme shows 60% activity of the natural tungsten protein [25]. The *Pyrococcus furiosus* AOR tungstoenzymes were recently shown to be able to incorporate Mo, albeit with no activity [26]. Given the high similarity of the two elements it is interesting to understand how biological systems have developed solutions to discriminate between the two. Significant advances have been made in the study of tungsten uptake by the cell, through the identification of selective transporters, the TupABC [27] and WtpABC [28] systems, but comparatively little is known about the intracellular regulation of protein expression in response to the two metals [15, 17].

Both molybdenum and tungsten FDHs have been reported in sulfate-reducing bacteria (SRB). These ancient organisms live in sulfide-rich environments, where molybdenum availability may be lower than tungsten, due to the very low solubility of molybdenum sulfides [29]. Nevertheless, two Mo-FDHs have been reported in SRB, from *Desulfovibrio vulgaris* Hildenborough [30] and *Desulfovibrio desulfuricans* ATCC27774 [31]. Both these enzymes are trimeric proteins that include the catalytic molybdopterin α subunit, the iron-sulfur electron transfer β subunit and a tetraheme cytochrome *c*. In contrast, a dimeric αβ W-containing FDH was isolated from *Desulfovibrio gigas* [32]. This W-FDH was the first tungstoprotein from a mesophile to have its structure determined [11]. Interestingly, *D. gigas* W-FDH was purified from cells not depleted of molybdenum, and from which a Mo-containing AOR was also isolated [32]. In addition, a dimeric FDH, homologous to the *D. gigas* W-FDH, was isolated from *Desulfovibrio alaskensis* cells grown in a rich medium (Postgate medium C) without supplementation of either metal, and shown to incorporate both Mo and W [17]. The first SRB genome to be sequenced, from *D. vulgaris* Hildenborough [33], revealed that this organism has three selenocysteine-containing FDHs. Analysis of gene organization indicates that FDH-1 (DVU0587-DVU0588) is a periplasmic dimeric protein (here referred to as FdhAB) homologous to the *D. gigas* W-FDH; FDH-2 is a periplasmic-facing oligomeric protein in which the αβ subunits associate with two cytochromes *c* and a membrane protein (DVU2481-DVU2482; here referred to as

FdhM for membrane-associated); and FDH-3 (DVU2811-DVU2812-DVU2809) is the trimeric periplasmic protein in which the αβ subunits associate with a tetraheme cytochrome c_3 (here referred to as $FdhABC_3$), which was reported to be a Mo-protein [30]. In this work we addressed the role of the molybdenum and tungsten metals on the relative expression of the three FDHs in *D. vulgaris*, and show that different isoenzymes are expressed in the presence of either metal.

2.3. Materials and methods

2.3.1. Culture media, growth conditions and preparation of cellular extracts

D. vulgaris Hildenborough was grown in Postgate medium C [34] or Widdel-Pfenning (WP) defined medium [35]. Postgate medium C contains 1g/L of yeast extract and is supplemented only with iron at a concentration of 25μM. Molybdate or tungstate were added to a final concentration of 0.1μM. In WP medium molybdate or tungstate were added separately from the other trace elements to a final concentration of 0.4μM. In each case, different electron donors were used (formate, lactate or hydrogen) at a final concentration of 40mM, with sulfate as electron acceptor (38 mM in Postgate medium C and 28mM in WP medium). When hydrogen or formate were used as electron donors, acetate (20mM) was also

included. Growth with hydrogen in WP medium with tungstate required the presence of trace amounts of molybdate (6 nM added).

Growth with formate or lactate was performed in 1L closed flasks containing half the volume of medium and a gas phase of 100% N_2 for Postgate medium C and 80% N_2/20% CO_2 for WP medium. Growth with hydrogen was performed in a 3L bioreactor with a continuous flow of 80% H_2/20% CO_2 at 500ml/min and stirring at 250rpm at constant pH of 7. In all cases the cells were grown at 37ºC. Cells were collected at mid-log phase and the different cellular extracts were obtained as described elsewhere [36].

2.3.2. Activity and kinetic assays

Formate dehydrogenase activities of cell extracts were performed as described before [36]. The assays were performed in a stirred cell in 50mM Tris-HCl pH7.6, inside the anaerobic chamber, with the enzymes pre-reduced with formate as described in [37]. For the kinetic assays with the pure enzymes the concentration of each FDH used was FdhAB: 0.0124nM, FdhM: 0.48nM and $FdhABC_3$: 0.25nM. The formate concentrations used were: 2µM, 5µM, 25µM, 50µM, 250µM and 500µM. Formate oxidation was measured following benzyl viologen (Sigma) reduction at 555nm. CO_2-reductase activity with the pure enzymes was performed as described in [38]. Each experiment was repeated at least three times.

2.3.3. Sulfate reduction rates

Cells were grown in medium C without addition of Mo or W with formate, lactate or hydrogen as electron donors. At mid-log phase cells were collected and transferred to fresh culture medium, and samples were taken for sulfate quantification during 3 hours. Sulfate quantification was performed by HPLC analysis with indirect UV detection at 310 nm, with a PRP-x100 column (Hamilton) and a mobile phase of 3% (v/v) methanol and 97% (v/v) of 6mM hydroxybenzoic acid (pH 10).

2.3.4. Gel electrophoresis

Non-denaturing polyacrilamide gels were stained for FDH activity with 2,3,5-triphenyltetrazolium chloride as described elsewhere [36]. Purified proteins were analyzed with 12% SDS-PAGE stained with a Comassie blue solution (0.1 %). For heme visualization gels were treated with a 10% trichloroacetic acid (TCA) solution and incubated in a DMB (3,3'-Dimethoxybenzidine dihydrochloride) solution [39].

2.3.5. Protein and metal quantification

The protein content of cell extracts was determined by the Bradford method (Sigma) with bovine gamma-globulin as standard (BioRad). The concentration of pure proteins was determined by the bicinchoninic acid using the BCA Protein Assay kit (Novagen) with bovine serum albumine as standard. Molybdenum and tungsten were

quantified by Inductively Coupled Plasma Mass Spectrometry (ICP-MS).

2.3.6. Western-blot analysis

Samples were run in a 10% SDS polyacrilamide gel and transferred to 0.45µm polyvinylidene difluoride (PVDF) membranes (Roche) for 1h at 100mV and 4ºC in a Mini Trans-Blot Electrophoretic Transfer Cell (BioRad). The membranes were equilibrated with a Tris-buffered saline solution (10mM Tris-HCl pH7.6, 150mM NaCl) and then treated with antiserum raised against artificial peptides of each *D. vulgaris* FDH (Davids Biotechnologie GmbH). Immunodetection of bound antibodies was done by treatment with anti-rabbit immunoglobulin G alkaline phosphatase conjugate (Promega) diluted 1:10000, followed by treatment with a solution of nitroblue tetrazolium salt (NBT) and 5-bromo-4-chloro-3-indolyl phosphate toluidine salt (BCIP) dissolved in 100mM Tris pH9.5, 100mM NaCl and 5mM $MgCl_2$ buffer.

2.3.7. Quantitative Real-Time PCR

FDH gene expression changes were monitored by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) through the amplification of the *fdhB* gene that encodes the small subunit of each protein. *D. vulgaris* was grown in WP or medium C with 0.2 g/L of yeast extract, supplemented with Mo or W as described above, and with formate, lactate or hydrogen as electron donors. The cells from

three independent experiments were collected anaerobically at mid-log phase, and after centrifugation the pellet was immediately frozen in liquid nitrogen and later used for RNA extraction. Total RNA was obtained using RNeasy Mini Kit (Qiagen) and was treated with Turbo DNAfree (Ambion). cDNA synthesis from each RNA sample (1µg) was performed using 1st Strand cDNA Synthesis Kit for qRT-PCR (Roche).

Primers were designed to generate amplicons of 150bp for the β subunit genes of each FDH and the 16S rRNA gene (Table 2.1).

Table 2.1. Primers used in qRT-PCR to determine the relative expression of each *D. vulgaris* FDH.

Target	Primers sequence (5' – 3')
FdhAB ß-subunit	Forward: GCGGAAGTGAAGAAGACATA
	Reverse: AACCGTGCGAACAGTTGCT
Membrane Fdh ß-subunit	Forward: CAGGGCCGACATGCTTCAA
	Reverse: TTCCGCCACCATGTGCGAA
$FdhABC_3$ ß-subunit	Forward: CTTCGACCGTGTCTCCTCT
	Reverse: CATCGACCAGATGCGCCAT
16S RNA	Forward: CCCTATTGCCAGTTGCTACC
	Reverse: AAGGGCCATGATGACTTGAC

The qRT-PCR was conducted in a Light Cycler 1.5 Real-Time PCR System (Roche) using Light Cycler Fast Start DNA Master SYBR Green I (Roche). Relative standard curves were constructed for each *fdhB*

gene and for the 16S rRNA gene using triplicate serial dilutions of cDNA. The relative expression of the *fdhB* gene of FdhAB and $FdhABC_3$ in different growth conditions was calculated by the relative standard curve method as described in [40] using the 16S rRNA gene as a reference.

2.3.8. Protein purification

Cells grown in hydrogen/sulfate with either Mo or W were collected at exponential phase and broken in a French Press in anaerobic conditions. The soluble fractions obtained after centrifugation were used for further purification. All chromatographic steps were performed at 4ºC inside a Coy anaerobic chamber with an Argon/2% Hydrogen atmosphere. *Step1.* The soluble fraction from Mo or W supplemented cells was first purified in a Q-Sepharose High Performance 26/10 (Pharmacia) column equilibrated with 10mM Tris-HCl pH 7.6. A step gradient with 10mM Tris-HCl and 1M sodium chloride was performed. *Step2.* The fractions containing the FDH activity (one for Mo and two for W soluble extracts), were concentrated and further purified on the same column (Mo conditions) or in a Resource Q column (W conditions), using the same buffers as in step 1. *Step3.* The fraction with FDH activity (Mo conditions) was further purified in a Resource Q column. In W conditions a third step was not required.

2.3.9. N-terminal determination

The three purified FDHs were run in a 12% SDS polyacrilamide gel and transferred to a PVDF membrane (Roche) as described above. The membrane was stained with a 0.1% Comassie solution and destained with 40% methanol. The band corresponding to each FDH large subunit was used for N-terminal sequencing by the method of Edman and Begg using an Applied Biosystems 491 HT sequencer.

2.4. Results

2.4.1. Effect of Mo and W on *D. vulgaris* growth and FDH activities

Initial experiments with extracts of *D. vulgaris* cells grown with either Mo or W in medium C (lactate as electron donor) showed that different bands were detected in native gels stained for FDH activity in either condition. This preliminary result indicated an effect of metal supplementation on FDH expression and prompted further studies.

The growth of *D. vulgaris* was tested in Postgate medium C, with lactate, formate or hydrogen as electron donors in the presence of either Mo or W, with no striking differences being observed. In lactate/sulfate medium a small increase in growth rate and growth yield was observed in W versus Mo conditions. This effect was slightly more pronounced in formate/sulfate medium, but was not detected

upon growth with H_2 in a bioreactor (data not shown). Cells were collected at mid-log phase and the FDH activities were measured in both soluble and membrane extracts (Figure 2.1). For all growth conditions the FDH activity in the membrane extracts is much lower than that of the soluble extracts. This suggests a very low level of the putatively membrane-associated FdhM, and/or that its catalytic subunit does not remain associated with the membrane. Both these factors were observed in the subsequent experiments.

With lactate as electron donor the FDH activities are quite low (Figure 2.1A). A similar level of activity was observed for the soluble extract of Mo or W-grown cells, but a higher activity for the W-grown cells was measured in the membrane extract. Notably, when cells were grown with formate or hydrogen there was a very high increase in activity when Mo was replaced with W, and this effect was more pronounced in the soluble than in the membrane fraction. The higher activities of cells grown with formate, and particularly with hydrogen, are in line with the reported higher expression of the *D. vulgaris* FDH genes during growth with these electron donors *versus* lactate [36, 41]. For these two conditions we tested also simultaneous addition of both metals or no addition. When the medium is not supplemented with either Mo or W, growth with formate or H_2 is still observed, but the cellular FDH activities are much lower. Medium C contains 0.1% (w/v) yeast extract which supplies trace amounts of both metals (1.4nM Mo and 0.3nM W [16]). The low levels of Mo or W present in medium C are apparently enough to sustain growth with formate or

H_2, but they do not allow full FDH activity. When both metals are present simultaneously during growth, the FDH activity is not significantly different from that with tungsten alone, indicating that an antagonistic effect is not present.

The sulfate reduction rates of mid-log cells grown in the absence of Mo or W were also measured for comparison with the FDH activities. The results were 13.0±3.2 $U.g^{-1}$ cells in lactate, 6.4±0.8 $U.g^{-1}$ cells in formate and 11.4±2.4 $U.g^{-1}$ cells in hydrogen. Since four molecules of formate have to be oxidized to reduce one molecule of sulfate, these results indicate that in the absence of metals or in the presence of Mo the measured FDH activity in the cell extracts (Figure 2.1) is less than that expected from the observed sulfate reduction rates. In contrast, in the presence of W this activity is higher than expected with formate or hydrogen as electron donors, indicating that in these conditions the growth is not limited by the FDH activity.

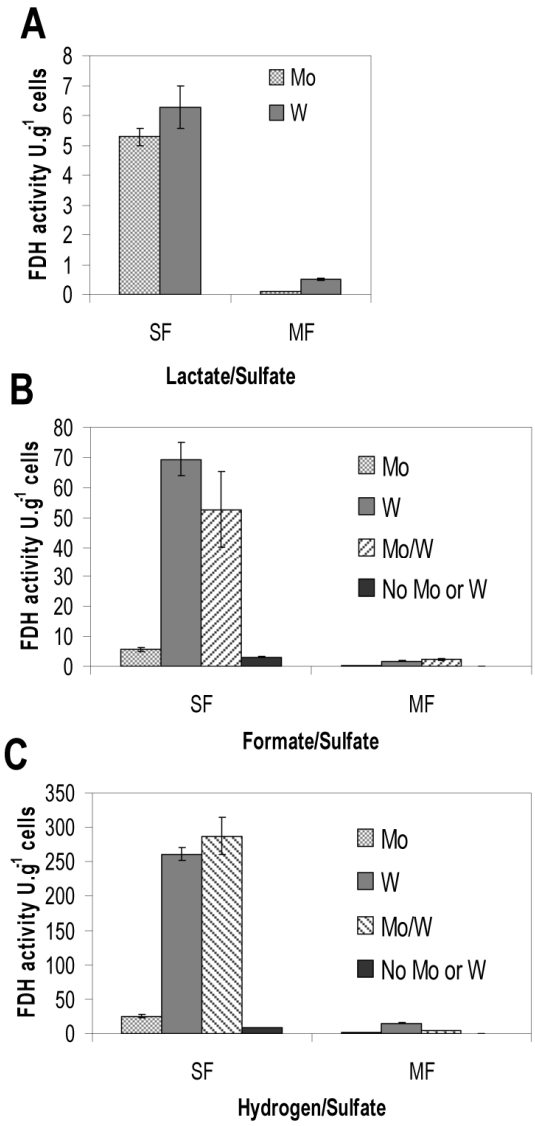

Figure 2.1. FDH activity in soluble fraction (SF) and membrane fraction (MF) of *D. vulgaris* cells grown with Mo (light grey), W (dark grey), both metals (stripes) or without either metal (black) added to the culture medium, and with different electron donors: **A-** Lactate, **B-** Formate or **C-** Hydrogen.

2.4.2. Analysis of FDHs by activity-stained native gels and Western-Blot

Analysis of cell extracts with native gels stained for FDH activity showed that one major band is detected for cells grown with Mo in the presence of any of the three electron donors (Figure 2.2). In extracts of cells grown with W two other bands are detected, of which band 2 is very intense in formate or hydrogen extracts. After purification of the three *D. vulgaris* FDHs (see below) it was possible to assign band 1 to $FdhABC_3$, band 2 to FdhAB and band 3 to the αβ subunits of FdhM. Thus, growth in the presence of W leads to a strong increase in activity of FdhAB (and to a less extent FdhM). To test whether this increase is due to specific incorporation of W in FdhAB or to an effect in protein levels, we performed Western blot analysis of cell extracts using polyclonal antibodies raised against specific peptides of the *D. vulgaris* FDHs. The immunoblots of cell extracts with anti-FdhAB antibodies show clearly that there is a significant increase in FdhAB levels when Mo is replaced with W in formate-grown cells, and this effect is even more pronounced in hydrogen-grown cells (Figure 2.3A). Similar high levels of FdhAB are observed when both Mo and W are present during growth, indicating that this enzyme is not repressed by Mo. Immunoblots of hydrogen-grown cell extracts with anti-FdhM and anti-$FdhABC_3$ antibodies show a small increase in the level of FdhM upon replacement of Mo for W, but no difference is detected in the level of $FdhABC_3$ (Figure 2.3B). In fact, the response level of $FdhABC_3$ antibodies is rather poor

(low titer), as observed from Figure 2.3B, where a low signal is observed in Mo conditions, even though the protein is known to be present from the activity-stained gels (Figure 2.2) and protein purification (see below). Thus, the Western results are not informative in the case of $FdhABC_3$.

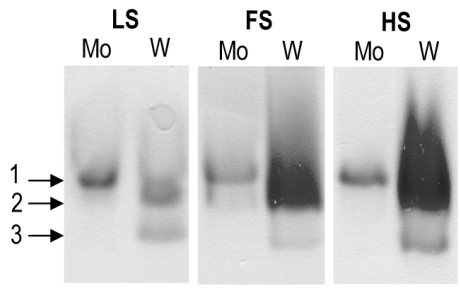

Figure 2.2. Non-denaturing polyacrylamide gels stained for FDH activity, of soluble extracts of *D. vulgaris* cells grown with Mo or W, and different electron donors. FS, formate/sulfate (70µg); HS, hydrogen/sulfate (60µg); LS, lactate/sulfate (70µg). The bands were identified after isolation of each FDH: **1.** $FdhABC_3$; **2.** FdhAB; **3.** αβ subunits of FdhM.

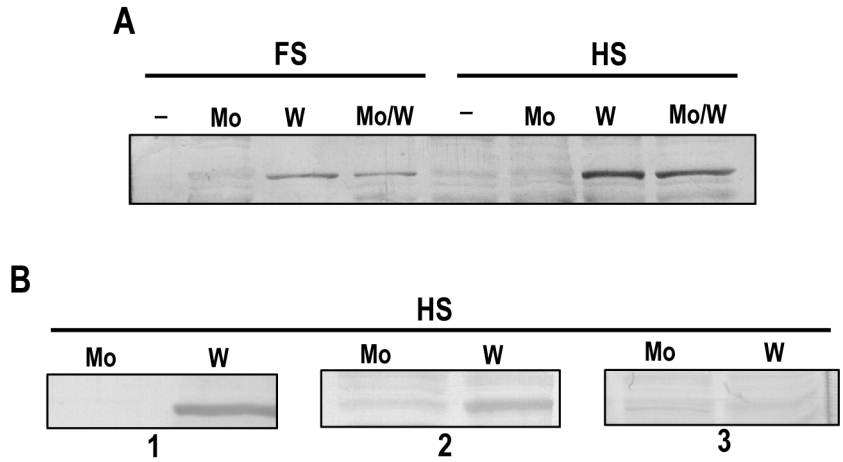

Figure 2.3. Western-blots of *D. vulgaris* soluble fractions using antibodies against **A-** FdhAB in cells grown with hydrogen/sulfate (HS) or formate/sulfate (FS); or **B-** FdhAB (1), FdhM (2) and $FdhABC_3$ (3) in cells grown with hydrogen/sulfate (HS). The culture medium used for cell growth was supplemented with only Mo, only W, both metals (Mo/W) or no metals (-). The amounts used for immunodetection were 75µg of soluble fraction for FdhAB antibodies and 100µg for Membrane Fdh and $FdhABC_3$ antibodies.

2.4.3. FDH gene expression analysis by qRT-PCR

The Western blot analysis shows a clear effect of tungsten in the protein levels of FdhAB and suggests that there is a metal regulation of FDH expression in *D. vulgaris*. To further test this we studied the consequence of replacing Mo for W in the mRNA levels of the *fdhB* gene encoding the small subunit of the FDHs by quantitative real time PCR. The results show clearly that the expression of FdhAB is higher in W *versus* Mo conditions, with either of the three electron donors (Figure 2.4). This effect is particularly dramatic in hydrogen-grown cells where a very high level of expression is observed with W,

and a very low level with Mo, in full agreement with the observations by activity-stained gels and Western blot. In lactate/Mo conditions the expression level of FdhAB is vanishingly small relative to W conditions, whereas with formate as electron donor, the difference in FdhAB expression between W and Mo conditions less pronounced. In fact, the difference in FdhAB transcript levels in formate conditions with Mo relative to W seems less than is observed at the protein level in the activity gels (Figure 2.2) and Western blot (Figure 2.3A). However, the mRNA levels are not necessarily reflected in protein levels. Thus, formate induces higher expression of this enzyme in the presence of both Mo and W, whereas hydrogen leads to a strong increase in expression only in the presence of W, suggesting that the interplay between regulation by the metals and the growth substrates is different for formate relative to lactate or H_2. Previous microarray experiments have already reported that formate and hydrogen lead to increased expression of the three FDHs in *D. vulgaris* relative to lactate [41, 42]. Overall, the qRT-PCR results for the FdhAB *fdhB* mRNA levels are in agreement with the protein levels and activity results, and suggest that tungsten is an inducer of FdhAB gene expression.

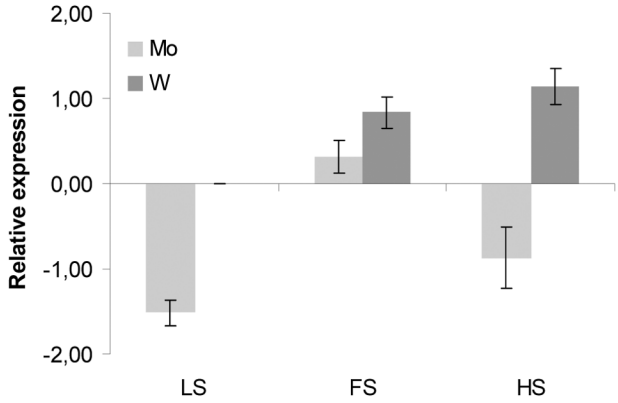

Figure 2.4. Relative expression of *D. vulgaris* FdhAB β-subunit gene determined by qRT- PCR, in cells grown with either Mo or W, and lactate/sulfate (LS), formate/sulfate (FS), or hydrogen/sulfate (HS). The $\log_{10}$ relative transcription values are shown in the y axis. The 16S rRNA gene was used as reference and the values are normalized with the LS/W condition. Results are from three independent biological experiments (mean±standard error).

A contrasting picture is observed for $FdhABC_3$. The *fdhB* mRNA levels of this enzyme indicate a reduced expression when Mo is replaced by W in the three growth conditions (Figure 2.6). This effect is more pronounced in formate-grown cells, for which a high level of expression of $FdhABC_3$ in Mo-conditions is observed. This effect was not apparent in the activity-stained native gels (Figure 2.2), but the intensity of the bands cannot be compared among different gels as it varies with time and this was not controlled. For this reason the soluble extracts of cells grown in the presence of Mo were ran simultaneously in the same gel (Figure 2.5). This confirmed an

increased activity of $FdhABC_3$ in formate/Mo conditions relative to the standard lactate/Mo condition. Thus, the qRT-PCR results show that the presence of tungsten/absence of molybdenum leads to a decrease of $FdhABC_3$ expression. Relative to lactate, formate induces expression of this enzyme, but not hydrogen, for which low levels are observed even with Mo. No reproducible results could be obtained with FdhM.

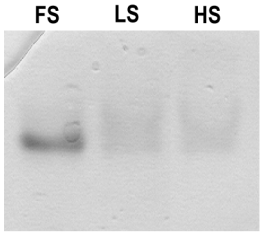

Figure 2.5. Non-denaturing polyacrylamide gel stained for FDH activity of soluble extracts of *D. vulgaris* cells grown with Mo and different electron donors. FS, formate/sulfate (80µg); LS, lactate/sulfate (80µg); HS, hydrogen/sulfate (60µg).

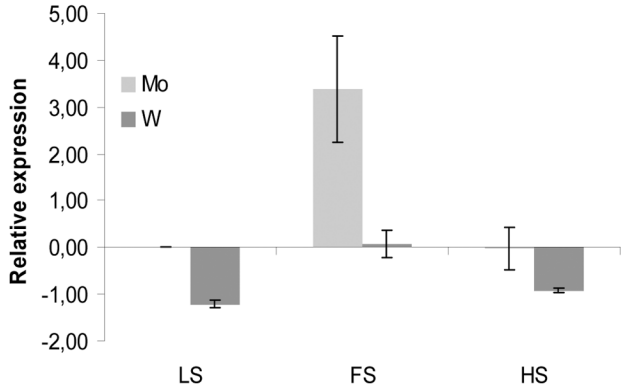

Figure 2.6. Relative expression of *D. vulgaris* $FdhABC_3$ β-subunit gene determined by qRT- PCR, in cells grown with either Mo or W, and lactate/sulfate (LS), formate/sulfate (FS), or hydrogen/sulfate (HS). The $\log_{10}$ relative transcription values are shown in the y axis. The 16S rRNA gene was used as reference and the values are normalized with the LS/Mo condition. Results are from three independent biological experiments (mean±standard error).

2.4.4. Purification of the three FDHs and metal analysis

Since only one FDH has been reported from *D. vulgaris* Hildenborough [30], it was of interest to purify and characterize the other two FDHs present in this organism, and determine their metal content. For this process, we used cells grown with hydrogen as electron donor in medium C supplemented with either Mo or W, since this is the electron donor leading to higher FDH expression. The two purifications (Mo and W conditions) were carried out separately, in anaerobic conditions, following the FDH activity. From Mo-grown cells only one peak of activity was detected after the first ion-exchange chromatography of the soluble fraction. Further purification led to the isolation of a single protein displaying three bands on SDS-gel (Figure 2.7A). The smaller band stained poorly with Coomassie, but was well visible after heme staining. This indicated the presence of hemes *c* (also visible in the UV-Vis spectrum), suggesting that the protein isolated under these conditions was $FdhABC_3$, which was

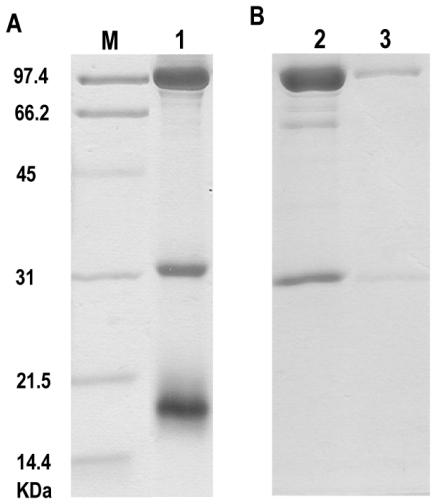

Figure 2.7. SDS-PAGE of FDHs purified from *D. vulgaris* soluble fraction stained with both Coomassie blue and heme staining (**A**), or just Coomassie blue (**B**). **M**, molecular markers; **1**- $FdhABC_3$ isolated from Mo/hydrogen grown cells; **2**- FdhAB and **3**- αβ subunits of FdhM, both isolated from W/hydrogen grown cells.

confirmed by N-terminal sequencing of the large subunit (YAVKL). From W-grown cells two peaks of activity were detected after the first ion-exchange chromatography of the soluble fraction. Further purification of the major peak led to the isolation of a protein with only two subunits by SDS-PAGE (Figure 2.7B), and N-terminal sequencing of the larger subunit (ELQKL) identified this protein as FdhAB. From the minor peak a two-subunit protein was isolated, which from N-terminal sequencing of the larger subunit (AELKI) corresponds to the αβ subunits of FdhM. This indicates that these two subunits of FdhM do not form a strong complex and dissociate from the membrane-bound protein and the two cytochromes *c* encoded in the same gene locus. Thus, protein purification gave further confirmation that $FdhABC_3$ is the major FDH expressed in *D. vulgaris* Mo-grown cells, whereas FdhAB and FdhM are the main FDHs present in W-grown cells.

Quantification of molybdenum and tungsten in the FDHs isolated from cells grown in medium C revealed that $FdhABC_3$ contained only Mo (Table 2.2), whereas FdhAB contained nearly equimolar amounts of Mo and W, as previously reported for *D. alaskensis* FdhAB [43]. Metal analysis of FdhM was not possible due to the very low levels of protein obtained. Since medium C is a rich medium containing low amounts of Mo and W, we repeated the purification of FdhAB and $FdhABC_3$ from cells grown in the same conditions as above, but in defined WP medium to which either Mo or W were added. However, growth with hydrogen in defined

medium with W required the presence of a low concentration of Mo (6 nM added). In these conditions, metal analysis by ICP-MS revealed that $FdhABC_3$ again incorporated only Mo (from a hydrogen/Mo medium) (Table 2.2), whereas FdhAB had a metal content of 80% W (from a hydrogen medium containing 0.4 µM W and 6 nM Mo). These results show that $FdhABC_3$ specifically incorporates Mo, whereas FdhAB can incorporate both metals.

Table 2.2. Mo and W quantification in FdhAB and $FdhABC_3$

Protein	mol W / mol protein	mol Mo / mol protein
FdhAB[a]	1.0±0.20	0.8±0.16
$FdhABC_3$[a]	0.03±0.006	0.7±0.14
FdhAB[b]	0.8±0.12	0.25±0.06
$FdhABC_3$[b]	n.d.	0.8±0.08

[a]Purified from cells grown in medium C. [b]Purified from cells grown in defined medium (WP). n.d. not detected.

Kinetic characterization of the three isolated FDHs (Table 2.3) showed that $FdhABC_3$ followed Michaelis-Menten kinetics with a turnover number of 262 s^{-1} and a K_M of 8 µM for formate. The FdhAB (isolated from medium C, and containing ~50% Mo and W) displayed a much higher turnover number (3684 s^{-1}) and also a lower K_M (1 µM). Thus, *D. vulgaris* FdhAB is an enzyme with a significantly higher

catalytic efficiency (k_{cat}/K_M of 3684 $\mu M^{-1}.s^{-1}$) than $FdhABC_3$ (k_{cat}/K_M of 33 $\mu M^{-1}.s^{-1}$). FdhAB isolated from cells grown in defined medium exhibited a lower level of activity, but this could have been the result of a harsher purification protocol. Since its metal content was also not 100% W, we cannot derive any conclusions as to whether the metal has an effect on the activity of the enzyme. Further studies will have to be carried out to clarify this point.The αβ subunits of FdhM showed a low turnover number of 81 s^{-1} and a K_M of 4 μM for formate. However, this low *in vitro* activity may not actually reflect the i*n vivo* situation, as it may result from the improper quaternary arrangement.

Table 2.3. Kinetic characterization of FDHs isolated from *D. vulgaris*

Protein	Specific activity ($U.mg^{-1}$)	Turnover number (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}.s^{-1}$)
FdhAB	903	3684	1	3684
FdhM	367	81	4	20
$FdhABC_3$	77	262	8	33

Several W-Fdhs have been reported to reduce CO_2 [38, 44], and given the lower redox potential of the W^{IV}/W^{VI} couple compared to Mo^{IV}/Mo^{VI} it was proposed that all CO_2-reductases are W-containing reversible FDHs, with Mo-containing enzymes probably operating only in the direction of formate oxidation [38]. We tested the CO_2-

reduction activity of the isolated FDHs and, contrary to the expectation, we could detect no activity for the W-induced FdhAB and FdhM enzymes, but a low level of activity was observed for the Mo-containing $FdhABC_3$ (1 $U.mg^{-1}$). This reveals that CO_2 reduction is not limited to W-containing FDHs and that even a Mo-containing FDH can act reversibly, even if with considerably lower activity for CO_2 reduction than formate oxidation.

2.5. Discussion

D. vulgaris Hildenborough genome encodes three FDHs, which are all in the periplasm, and thus are likely to operate as formate-uptake enzymes contributing to the proton motive force. Two of them ($FdhABC_3$ and FdhAB) are believed to transfer electrons to the cytochrome c_3 pool [33, 45], similarly to the four periplasmic hydrogenases, whereas the third FDH is associated with a membrane subunit that may allow direct reduction of the menaquinone pool. The $FdhABC_3$ from *D. vulgaris* Hildenborough has been reported to be a Mo-containing protein [30], whereas the FdhAB enzyme from the closely related *D. gigas* was reported to be a W-protein [32]. In addition, the genome of *D. vulgaris* Hildenborough encodes also for a Mo/W ModABC transporter, and a W-specific TupABC transporter.

In this work we studied the effect of Mo and W in the relative expression of the three *D. vulgaris* FDHs. There were no significant differences between growth with either Mo or W when lactate,

formate or hydrogen were used as electron donors. However, growth with W led to a much higher FDH activity in formate, and especially in hydrogen-grown cells, whereas only a small difference was observed in lactate-grown cells. Using native gels stained for FDH activity we observed that different FDH isoenzymes are expressed during growth with either Mo or W, suggesting that a metal dependent regulatory mechanism is present. This effect was confirmed by Western blot, qRT-PCR and protein isolation, which showed that the level of the $FdhABC_3$ and FdhAB enzymes depends on the metal present, besides being affected by the electron donor as previously reported [41, 42]. The presence of tungsten and absence of molybdenum induces the increase of FdhAB and reduces the level of $FdhABC_3$, with either of the three electron donors. Activity-stained native gels and Western blot suggest that this condition also leads to an increase of FdhM, although this could not be confirmed by qRT-PCR. In cells grown in the presence of Mo, the $FdhABC_3$ is the main FDH present, and FdhAB is only expressed significantly with formate. In the presence of W FdhAB is the main FDH, and FdhM is present at a low level. Overall, the results indicate that $FdhABC_3$ is the main FDH in formate/Mo-conditions, and FdhAB in H_2/W-conditions.

These results provide the first direct evidence of transcriptional/post-transcriptional control of FDH isoenzymes performed by Mo and W. Previous reports have indicated that similar mechanisms are present in other organisms, and with another Mo/W enzyme. In *Methanococcus vannielii* a single FDH was reported when

cells were grown in the presence of tungsten, whereas two enzymes were observed in its absence [46]. In *Syntrophobacter fumaroxidans*, a syntrophic acetogenic bacterium that can also grow by reduction of sulfate or fumarate, two W-containing FDHs have been isolated [38], and several others are encoded in the genome [47]. In this organism, growth in the presence of W also leads to a strong increase in total FDH activity relative to growth with Mo, either during syntrophic growth with a methanogen or during growth with propionate and fumarate [48]. In the presence of both metals a decrease in FDH activity is observed for the pure culture relative to the W-supplemented culture, suggesting an antagonist effect of Mo in a W-FDH, whereas such effect is not observed for the co-cultured cells, which indicates the involvement of different FDHs. In the pathogen *Campylobacter jejuni*, which also has both ModABC and TupABC transporters, its single FDH was shown to be active with either metal but to display a preference for tungsten, with the TupABC system acting as a specific transporter for W to be incorporated in FDH [49, 50]. In this organism a ModE-like regulatory protein represses the *mod* operon in the presence of both Mo or W and the *tup* operon only in the presence of W.

A previous example of two isoenzymes incorporating either Mo or W with differential metal regulation has been reported for FMDHs from *Methanobacterium wolfei* and *Methanobacterium thermoautotrophicum* [51]. The two isoenzymes have different subunit composition and the tungsten protein (Fwd) is constitutively

expressed, whereas the one containing molybdenum (Fmd) is induced by molybdate. Like *D. vulgaris* FdhAB, the Fwd protein can also incorporate Mo. Curiously, both proteins share one of the subunits, FwdA, encoded in the *fwd* operon.

The understanding of Mo/W-dependent gene regulation is still rather limited, and studies have focused mainly on regulation of molybdate transport by Mo, and not on intracellular mechanisms [15, 52]. Nevertheless, a widespread bacterial riboswitch was recently identified, which can sense Moco (molybdopterin cofactor) in the cell, and control gene expression of molybdate transporters, Moco biosynthesis enzymes and some proteins that use Moco as a cofactor [53]. Interestingly, this RNA can distinguish between Moco and Tuco (tungstopterin cofactor), and a second group of closely related RNAs were identified in bacteria that use tungsten, and were proposed to be Tuco riboswitches [53]. We could not identify either of these riboswitch sequences close to the genes for *D. vulgaris* FDHs. So, at this stage, it is difficult to speculate on the mechanism of regulation that can discriminate between Mo and W. The recent report of low Mo incorporation in the *P. furiosus* tungsten AORs, when the organism is grown with very low W and high Mo concentrations, reveals that also in this organism a selective intracellular mechanism is present to discriminate between the two metals, even when high intracellular concentrations of Mo are present in this tungsten-dependent organism [26].

Our results show that in *D. vulgaris* the incorporation of Mo or W at the active site of FDHs is regulated not only at the level of gene expression of the different isoenzymes, but also by their different selectivities in metal incorporation. For $FdhABC_3$ a high selectivity for Mo incorporation is observed, which is paralleled by a strong decrease in protein level in the absence of Mo/presence of W. In contrast, the FdhAB enzyme can incorporate either metal, even when grown with much higher concentrations of W, indicating that the process of metal incorporation is not so specific or tightly regulated. This enzyme has a much higher catalytic efficiency than $FdhABC_3$, but is only detected if W is available, suggesting an overall preference of the organism for W. The ability of *D. vulgaris* (and probably other SRB) to use both tungsten and molybdenum, and to regulate FDH expression according to their levels, has significant environmental implications. SRB are important players in the degradation of organic matter in anaerobic habitats, namely involving syntrophic associations where formate and hydrogen are key intermediates [1, 2, 47]. If sulfate is available, the activity of SRB will lead to sulfide formation and metal sulfides precipitation. The higher solubility of tungsten *versus* molybdenum sulfides may change the relative abundance and bioavailability of these metals, and thus the capacity to use both of them increases the metabolic versatility and is likely to confer a selective advantage to the organism. A similar versatility is observed in *D. vulgaris* regarding the periplasmic uptake hydrogenases, where three different enzymes are expressed in

response to Ni and Se availability [54]. Thus, adaptation to trace element availability seems to be a fitness factor in *D. vulgaris*.

2.6. Acknowledgments

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Chapter 3

Function of formate dehydrogenases in *Desulfovibrio vulgaris* Hildenborough energy metabolism

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3.1. Summary

Desulfovibrio vulgaris Hildenborough genome encodes three formate dehydrogenases, two of which are soluble periplasmic enzymes (FdhAB and $FdhABC_3$) and one that is periplasmic but membrane-associated (FdhM). The FdhAB and $FdhABC_3$ were recently shown to be the main formate dehydrogenases present during growth with lactate, formate or hydrogen. To address the role of these two formate dehydrogenases in D. vulgaris metabolism, mutants for FdhAB and $FdhABC_3$ were generated (*ΔfdhAB* and *Δ$fdhABC_3$*, respectively), and the effect of the mutations was studied in various growth conditions. Both soluble formate dehydrogenases are important for growth on formate in the presence of Mo, whereas in W only the FdhAB plays a role, due to the repression of *$fdhABC_3$*. Both *ΔfdhAB* and *Δ$fdhABC_3$* display defects in growth with lactate/sulfate providing evidence for the involvement of formate cycling in this process. In contrast, both mutants grew similarly to the wild-type in hydrogen/sulfate. In the absence of sulfate, the D. vulgaris cells produced formate when supplied with H_2/CO_2, which resulted from CO_2 reduction by the periplasmic enzymes, as evidenced by the reduced accumulation of formate in the mutants. We propose this may be an expression of the ability of some sulfate reducing bacteria to grow by hydrogen oxidation coupled to CO_2 reduction in syntrophy with organisms that consume formate and are less efficient in H_2 utilization.

3.2. Introduction

Formate is an important metabolite in anaerobic ecosystems, formed by fermentative organisms in the degradation of complex organic molecules. It is a common growth substrate for many bacterial and archaeal microorganisms, and a cellular intermediate in several processes like methanogenesis, acetogenesis or methylotrophy [1-4]. Together with hydrogen, it is also involved in interspecies electron transfer by syntrophic communities of bacteria and archaea in methanogenic or sulfate-reducing environments [2, 5, 6].

The ability to use formate depends on formate dehydrogenases (FDH), enzymes that catalyze the reversible conversion of formate to CO_2. Reflecting the different physiological roles in which formate participates, the FDHs display large diversity in quaternary structure, cofactor composition, electron donor/acceptor and cellular localization [7, 8]. Most formate dehydrogenases contain a molybdenum (Mo) or tungsten (W) pterin cofactor, but some aerobic bacteria possess NAD^+-dependent FDHs that lack a prosthetic group [7, 9, 10].

The FDHs that function mainly as CO_2-reductases are cytoplasmic enzymes described in acetogens and fermentative organisms, where they produce formate in the first step of the Wood-Ljungdahl pathway for the biosynthesis of C1 compounds [11]. A NADPH-dependent FDH was first described in the acetogen *Moorella thermoacetica* [12]. In the acetogen *Treponema primitia*, a

thermite gut symbiont, it was recently shown that the enzyme responsible for CO_2 reduction is associated with an [FeFe]-hydrogenase as its putative electron donor [13]. This is possibly an adaptation of the organism to a very H_2-rich environment, and allows formate production directly from H_2.

Formate can be used as substrate by some methanogenic Archaea, where it is oxidized to CO_2 to be used in CH_4 formation. The cytoplasmic soluble FDHs use the F_{420} coenzyme as electron acceptor for formate oxidation [14-16]. In addition, it was recently shown that in *Methanococcus maripaludis* a cytoplasmic FDH can transfer electrons directly to heterodisulfide reductase in an electron bifurcation process that is coupled to ferredoxin reduction [17].

Cytoplasmic FDHs, like FDH-H in Escherichia coli, can also be associated with a hydrogenase forming a formate hydrogen-lyase (FHL) complex that is membrane-bound [18]. FDH-H is expressed during *E. coli* fermentative growth, and it oxidizes formate to CO_2, coupled to reduction of protons to H_2 by the hydrogenase partner. A soluble FHL complex has also been described in *Eubacterium acidaminophilum* [19], and it is also deduced from the genomic analysis of several organisms such as sulfate-reducing bacteria (SRB) [20] and syntrophic bacteria [21, 22]. In the SRB it is not clear in which direction it operates, or it likely can operate both ways depending on the relative concentrations of formate, CO_2 and H_2.

Periplasmic FDHs act generally to oxidize formate and are usually membrane-bound through an integral membrane subunit

that transfers electrons to the quinone pool [23, 24]. In most microorganisms this subunit is a b-type cytochrome that may be involved in a redox loop mechanism with another membrane-bound enzyme, resulting in electron transfer to the cytoplasm and proton transfer to the periplasm, thus contributing to the generation of proton motive force and, consequently, energy conservation [25]. However, in the deltaproteobacterial sulfate reducers most periplasmic FDHs (and hydrogenases) are soluble, and transfer electrons to cytochromes c [20, 26]. These FDHs either include a dedicated cytochrome c_3 subunit (FdhABC3) [27, 28], or they include only two subunits (FdhAB) [29, 30], and transfer electrons to the periplasmic c_3 network [31] and from these to membrane-bound complexes [32]. A two subunit periplasmic FDH was also isolated from *Syntrophobacter fumaroxidans*, a propionate oxidizing syntrophic bacterium also able to reduce sulfate [33].

In *Desulfovibrio vulgaris* Hildenborough three periplasmic FDHs are present, two of which are soluble ($FdhABC_3$ and FdhAB), and one is associated with the membrane through a NrfD-type protein [28, 34]. No cytoplasmic FDHs are encoded in the genome. The $FdhABC_3$ and FdhAB are the two main FDHs detected in D. vulgaris, and they are differentially regulated by the metals Mo and W [34]. Mo induces expression of the $FdhABC_3$ enzyme, which specifically incorporates Mo, and represses expression of FdhAB. In contrast, W induces expression of FdhAB, which is a W-enzyme but that can also incorporate Mo, and represses $FdhABC_3$. As expected, increased

expression of both Fdhs during growth with formate/sulfate was observed (relative to lactate/sulfate), as reported before [35]. In addition, a previous study revealed increased transcription of all *D. vulgaris* FDHs during growth with H_2/sulfate [36], and we observed a similar effect in particular for the W-FdhAB [34]. The function of FDHs during growth of *D. vulgaris* with H_2/sulfate is not immediately obvious, and two alternatives can be considered. In the first, formate is formed in the cytoplasm by the pyruvate-formate lyase (the Pfl activating enzyme is also upregulated during growth in H_2/sulfate [36]), and is then transported to the periplasm where it is oxidized by the periplasmic FDHs; transfer of the resulting electrons across the membrane for the cytoplasmic reduction of sulfate, while protons are left in the periplasm, contributes to the proton-motive for energy generation. This intracellular formate cycling has been proposed before for growth of *D. vulgaris* in lactate/sulfate [37]. The second alternative is that during growth in H_2/CO_2/sulfate one or more of the FDHs are acting to reduce CO_2 to formate. This could be oxidized in the periplasm by another FDH, or it could be transported inside the cell and converted to pyruvate by the reversibvle enzymes of C1 metabolism. *D. vulgaris* lacks some enzymes required for the complete reductive Acetyl-CoA pathway.

In order to get further insight on the function of the two soluble FDHs in D. vulgaris metabolism, we constructed deletion mutants for the FdhAB (*ΔfdhAB*) and the $FdhABC_3$ (*Δ*$fdhABC_3$), and

compared their phenotype to the wild type strain (wt) during growth with formate, lactate and H_2/CO_2 in the presence of either Mo or W.

3.3. Materials and methods

3.3.1. Construction of mutant strains

The 500 bp regions, located within and downstream of the gene encoding the catalytic subunit of $FdhABC_3$ (*fndG3*; DVU2812), were PCR-amplified using primer pairs p290f/p291r and p292f/p293r, respectively (Table 3.1). The amplified fragments were cloned sequentially into pNOT19 by digestion with *Sac*I and *Bam*HI, and *Pst*I and *Bam*HI and ligation to obtain pNOTΔfndG3. The latter was cleaved with *Bam*HI and ligated to the *cat* gene-containing 1.4 kb *Bam*HI fragment from pUC19Cm to obtain pNOTΔfndG3Cm. NotI digestion of the latter and insertion of the 4.2 kb *Not*I fragment from pMOB2 gave pNOTΔfndG3CmMob, which was transformed into *E. coli* S17-1. Following conjugation of *E. coli* S17-1 (pNOTΔfndG3CmMob) and *D. vulgaris*, single crossover integrants were obtained on medium E plates containing chloramphenicol (Cm), as described elsewhere [38]. Growth of the integrant in defined medium containing Cm and 5% (wt/vol) sucrose produced the double crossover mutant *D. vulgaris ΔfdhABC*$_3$ in which 1029 bp of the 3' end of the 3039 bp *fndG3* coding region were replaced with the cat gene.

Likewise, the 500 bp regions, located up- and downstream of the fdhAB genes (DVU0587 and DVU0588) were PCR-amplified using primer pairs p411f/p412r and p413f/p414r, respectively (Table 1) and cloned following digestion with *Hind*III and *Bam*HI and *Kpn*I and *Bam*HI, respectively. The procedures used were similar as for construction of the *ΔfdhABC$_3$* mutant, except that the *nptii* gene, encoding kanamycin (Km) and G418 resistance, was used as the selectable marker [39, 40]. This resulted in construction of the suicide plasmid pNOTΔFdhABKmMob and the double crossover mutant D. vulgaris *ΔfdhAB*, which had all of *fdhAB* replaced with the *nptii* gene.

Table 3.1. PCR primers used for mutant construction

Primer	Sequence
p290f	tcgagagctcTCAGCAGGCTGGCGACGTACT
p291r	tcgaggatccAGTTCGAGAAGGGACCCGACG
p292f	tcgaggattcGCGTCGAAGGTCGCCTTTCAG
p293r	tcgactgcagCTGTCAGGTCTGTCGCCGATG
p411f	tcgaaagcttAGCGCCTGACGCACCCTGTGA
p412r	tcgaggatccCGTGCCTCCTCTGGGGTTCAG
p413f	tcgaggatccGGTGGAGGGACCGGTAACGGT
p414r	tcgaggtaccAGTGCCGGACATCCTCCGGTC

3.3.2. Culture media, growth conditions and preparation of soluble fraction

D. vulgaris wt and mutants were grown in modified Postgate medium C [41] containing 0.2g/l of yeast extract, an iron concentration of 25μM, 1μM of nickel and selenium, and 38mM of sulfate. Electron donors used were lactate, formate and hydrogen added to a final concentration of 40mM in the case of organic acids. When hydrogen was the electron donor a mixture of 80% hydrogen/20% CO_2 was used as gas phase to a final pressure of 1bar. Acetate (10mM) was also present in formate or hydrogen media. A final concentration of 0.1μM of either molybdenum or tungsten was added to the growths, which were performed in anaerobic conditions in 500ml flasks half filled with culture medium. The cells were grown at 37ºC and optical density was determined spectrophotometrically (Shimadzu) at 600nm. All the density measurements are averages of two biologically independent experiments.

3.3.3. Analytical procedures

For metabolite analysis 1ml samples of culture were taken at several intervals, immediately centrifuged and the supernatant frozen at -20ºC for posterior analysis. Lactate, formate and acetate concentrations were determined by high performance liquid chromatography (HPLC), with a Waters chromatograph and a LKB 2142 Differential Refractometer (LKB Bromma) detector. An Aminex HPX-87H column (BioRad) was used at 60°C and data were collected

with the Millenium 32 software, version 3.05.01 (Waters). Samples (20µl) were eluted isocratically at a flow rate of 0.5mL.min^{-1}, with 0.005N of H2SO4. Sulfate concentration was determined with a PRP-x100 column (Hamilton) on a Waters Acquity Ultra-Performance Liquid Chromatograph with indirect UV detection at 310nm, and data were collected and processed by Empower 2 software (Waters). Samples (10µl) were injected at 25ºC and eluted isocratically at a flow rate of 2ml.min^{-1}, with a mobile phase of 3% (v/v) methanol and 97% (v/v) of 6mM hydroxybenzoic acid (pH10).

3.3.4. Formate quantification in cell suspensions

Cells grown with either lactate or H_2 were collected at mid-log phase, centrifuged and suspended in a 10-times smaller volume of fresh culture medium containing a limiting amount of sulfate (10mM). Lactate (40mM) or H_2/CO_2 (1bar) were provided as electron donors. Cells were incubated at 37ºC and samples taken at several intervals and treated as described above. For formate quantification, 20µl of supernatant from each sample were dispensed per well in a 96-well plate (Greiner). The reaction was started by adding a solution containing 1mM NAD^+, 40mM Tris-HCl buffer pH8 (at 25ºC) and 0.5U of Candida boidinii formate dehydrogenase (Sigma) to a final volume of 200µl (per well). Absorbance was read in the beginning of the reaction in a 96-well plate reader (BioTek), at 340nm and again after 1 hour incubation at 37ºC. When necessary the samples were appropriately diluted.

3.4. Results

In a previous study we showed that $FdhABC_3$ is the main Fdh present in Mo conditions, and is particularly upregulated during growth with formate/sulfate. The presence of W causes *fdhABC*$_3$ repression. On the other hand, FdhAB is the main FDH in W conditions, and *fdhAB* is particularly upregulated during growth with H_2/sulfate. The metals had no effect on growth rate during growth with H_2/sulfate, whereas a small increase in growth rate was observed on formate/sulfate and lactate/sulfate in W relative to Mo [34]. These results with wt cells were confirmed in the present work.

3.4.1. Growth in formate/sulfate

When formate (40mM) was used as electron donor with excess sulfate (38mM) significant differences were observed between wild-type and mutants, and different phenotypes were revealed by Mo or W (Figure 3.1). In the presence of Mo the wt and *ΔfdhABC*$_3$ strains had a similar growth rate, but the final cell density of the mutant was considerably lower. The *ΔfdhAB* strain had a much lower growth rate but the final cell density was similar to the *ΔfdhABC*$_3$ mutant (Figure 3.1, panel A). In the presence of W, *ΔfdhABC*$_3$ had a slightly lower growth rate than *wt*, but the final cell density was similar for both, while the growth of *ΔfdhAB* was severely impaired (Figure 3.1, panel D). Metabolite analysis showed that 4 molecules of formate were consumed for 1 of molecule of sulfate, as expected. Formate consumption and sulfate reduction follow the growth pattern in all

experiments (Figure 3.1, panels B, C, E and F), including for *ΔfdhAB* mutant in W conditions where practically no growth occurs.

D. vulgaris does not possess all the enzymes necessary for a complete Wood-Ljungdahl pathway, namely a formyltetrahydrofolate synthase, which would allow the use of CO_2 or formate as carbon sources for C1 metabolism [42]. For this reason, when growing with formate or H_2/CO_2 as electron donors, acetate has to be added as carbon source. Acetate is activated to acetyl-CoA, which can be reductively carboxylated to pyruvate [43], and is only used for anabolic reactions, with 70% of cell carbon being derived from acetate and 30% from CO2 [44]. Acetate was not detectably metabolized and remained constant (data not shown), probably due to the low biomass yield.

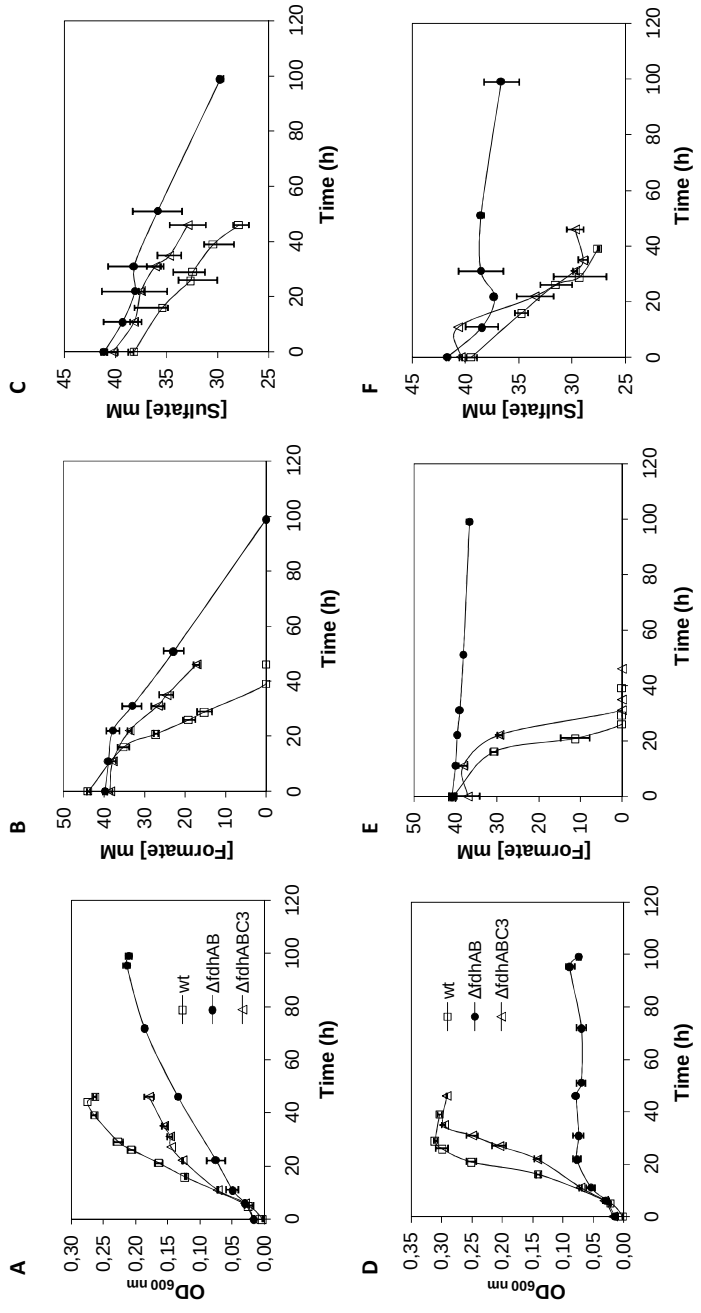

Figure 3.1. Growth curves (A, D) and metabolite production/consumption (B, C, E, F) of the *wt*, *ΔfdhAB* and *ΔfdhABC*$_3$ mutants in formate (40mM)/ acetate (10mM)/ sulfate (38mM) medium, in the presence of either Mo (0.1µM) (A, B and C) or W (0.1µM) (D, E and F). The symbols in panels A and D apply to the other panels. Results are means from duplicate experiments ± standard error.

3.4.2. Growth in lactate/sulfate

When lactate (40mM) was used as electron donor with excess sulfate (38mM) both mutants, *ΔfdhAB* and *ΔfdhABC$_3$*, grew at a lower rate than the *wt* and reached a lower cell density. Both effects were more pronounced in the *ΔfdhABC$_3$* strain, and this behavior was identical whether Mo or W were present in the medium (Figure 3.2, panels A and D). These results indicate that FDHs play a role in energy metabolism during lactate/sulfate growth. Metabolite analysis shows that in the wild-type 40mM lactate and 20mM sulfate were consumed (Figure 3.2, panels B, C, E and F), in accordance with the expected stoichiometry. The mutants were not able to metabolize all lactate present in the medium. Both mutants consumed 30mM of lactate and reduced 15mM of sulfate, in Mo or W conditions. Part of the lactate oxidized will be converted to acetate for energy generation and part will be incorporated as biomass [43]. In *D. vulgaris wt* cells, about 30mM lactate were converted to the same amount of acetate (in both Mo or W), while ca. 10mM were incorporated into biomass. A similar situation was observed for the mutants, with comparatively less lactate oxidized and less acetate being formed. No formate was detected during growth.

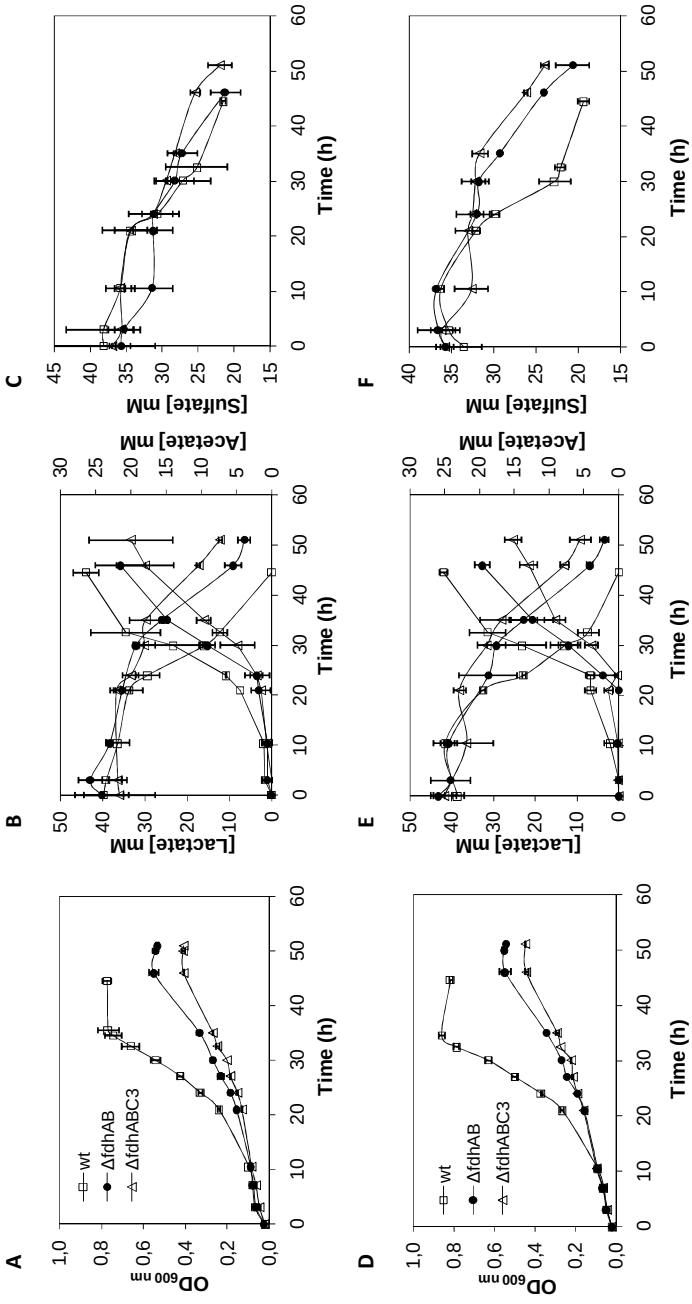

Figure 3.2. Growth curves (A, D) and metabolite consumption (B, C, E, F) of the *wt*, *ΔfdhAB* and *ΔfdhABC*$_3$ mutants in lactate (40mM)/ sulfate (38mM) medium, in the presence of either Mo (0.1μM) (A, B and C) or W (0.1μM) (D, E and F). The symbols in panels A and D apply to the other panels. Results are means from duplicate experiments ± standard error.

3.4.3. Growth in H_2/sulfate

When hydrogen was used as electron donor, a headspace pressure of 1bar of 80% H_2/20% CO_2 was maintained during growth and acetate was again present to be used as additional carbon source. Surprisingly, no significant differences in growth rate or cell densities were observed between wild-type and mutants, except for a small delay to start growth, and a slightly reduced cell density in the *ΔfdhABC*$_3$ mutant (Figure 3.3, panels A and C). This is contrary to what we had expected since hydrogen, leads to a strong increase in the levels of FdhAB in W conditions [34, 36]. However, the lack of either FdhAB or FdhABC$_3$ did not have a major impact on growth, whether Mo or W were present in the medium. Once more acetate remained stable at 10mM (data not shown) during growth, possibly because it is being recycled due to the large excess of CO_2. Approximately 10mM sulfate were consumed by all strains before growth stopped, which was likely due to inhibition caused by the accumulation of sulfide (Figure 3.3, panels B and D). As with lactate/sulfate, no formate was detected during growth.

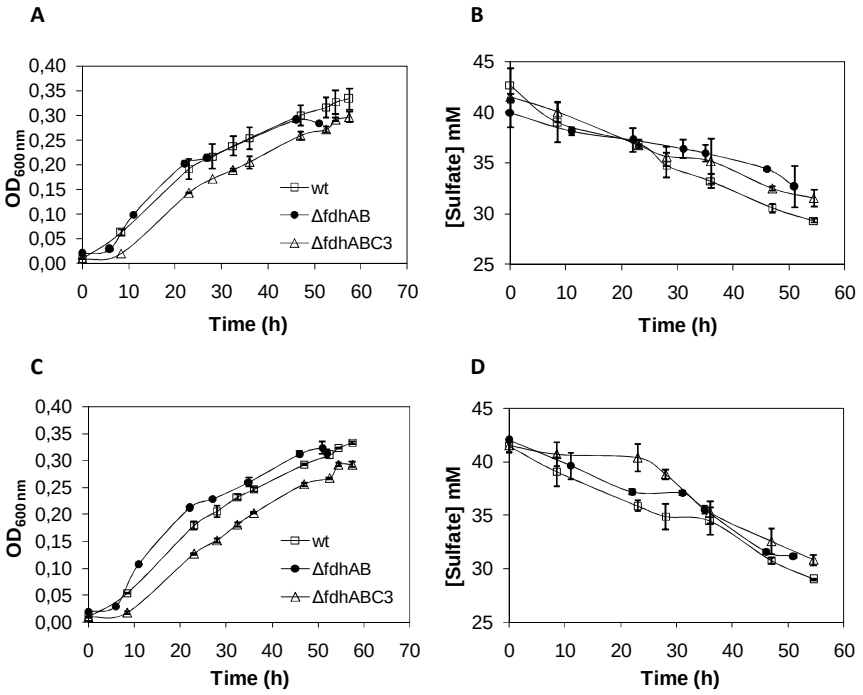

Figure 3.3. Growth curves (A, C) and sulfate consumption (B, D) of the *wt*, *ΔfdhAB* and *ΔfdhABC*$_3$ mutants in hydrogen/CO_2 (1bar)/ acetate (10mM)/ sulfate (38mM) medium, in the presence of either Mo (0.1µM) (A and B) or W (0.1µM) (C and D). The symbols in panels A and C apply to the other panels. Results are means from duplicate experiments ± standard error.

3.4.4. Formate quantification in cell suspensions

The phenotype of the FDH mutants during growth with lactate/sulfate suggests that intracellular formate cycling is occurring in those conditions. In contrast, no phenotype was observed for growth with hydrogen, which is a regulator for FdhAB expression. Despite formate not being detected in either growth condition, this does not mean that it is not being formed and consumed by the cells

at significant rates, with the excreted concentration being so low that is below the HPLC detection limit. In order to check this hypothesis we carried out experiments with concentrated cell suspensions, and measured formate enzymatically, which is a more sensitive method. In these experiments sulfate was present in limiting amounts (10mM).

With lactate as electron donor (40mM) sulfate was consumed in the first three hours of incubation by the *wt* and *ΔfdhABC3* mutant, whereas it took longer for the ΔfdhAB mutant to consume the entire sulfate (Figure 3.4). For 10mM sulfate, about 20mM of lactate were consumed and 20mM of acetate produced (data not shown), according to the expected stoichiometry. After sulfate was consumed a very low level of formate was detected transiently, but the values were very low and poorly reproducible. Nevertheless, these values were higher in the mutants than in the wt, which agrees with the FDHs acting to oxidize formate. When sulfate is depleted, lactate can be fermented, producing acetate, CO_2 and H_2 [37, 45], but formate could also be formed through PFL. Since H_2 is not being removed from the medium it will halt the fermentation for thermodynamic reasons.

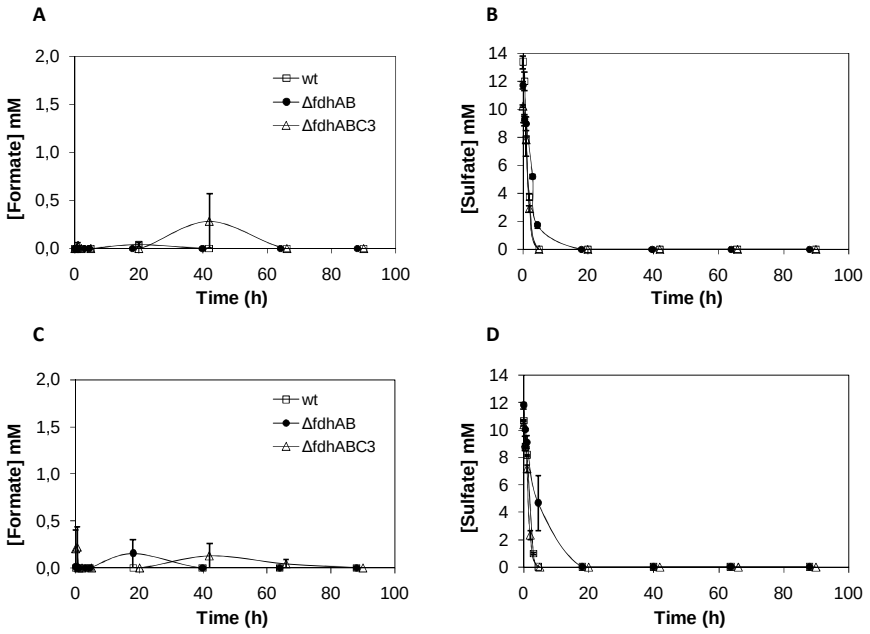

Figure 3.4. Formate production (A, C) and sulfate consumption (B, D) in wt, ΔfdhAB and ΔfdhABC3 cell suspensions incubated with lactate (40mM) and limiting sulfate (10mM), in the presence of Mo (A, B) or W (C, D). The symbols in panels A and C apply to the other panels. Results are means from duplicate experiments ± standard error.

With H_2 as the electron donor, sulfate took a few more hours to be consumed than with lactate but eventually it became depleted after 40 hours incubation in the wild-type and mutants in Mo, and somewhat less in W (Figure 3.5, panels B and D). In the Mo condition, a low level of formate started to be detected at 20h incubation, both in *wt* and *ΔfdhAB* cells, and later at 40h in *ΔfdhABC3* cells (Figure 3.5, panel A). After sulfate was depleted, formate started to accumulate, and at the end of the incubation period the formate concentration was higher for the wt (10mM) than for both mutants, suggesting that

FdhAB and FdhABC$_3$ were functioning as CO_2-reductases. The rate of CO_2 reduction was similar for *wt* and *ΔfdhABC$_3$*, but was considerably slower for the *ΔfdhAB* mutant. *ΔfdhABC$_3$* cells accumulated more formate than *ΔfdhAB* (8 and 5mM, respectively). In the W condition, formate started accumulating at 20h incubation for both wt and *ΔfdhABC$_3$*, and proceeded with a similar rate in both (Figure 3.5, panel C). At the end of the incubation there were 11mM formate for the *wt* and 10mM for *ΔfdhABC$_3$*. In contrast, practically no formate accumulated in the incubation of the *ΔfdhAB* cells, with only 0.4mM being detected at the end of the incubation period.

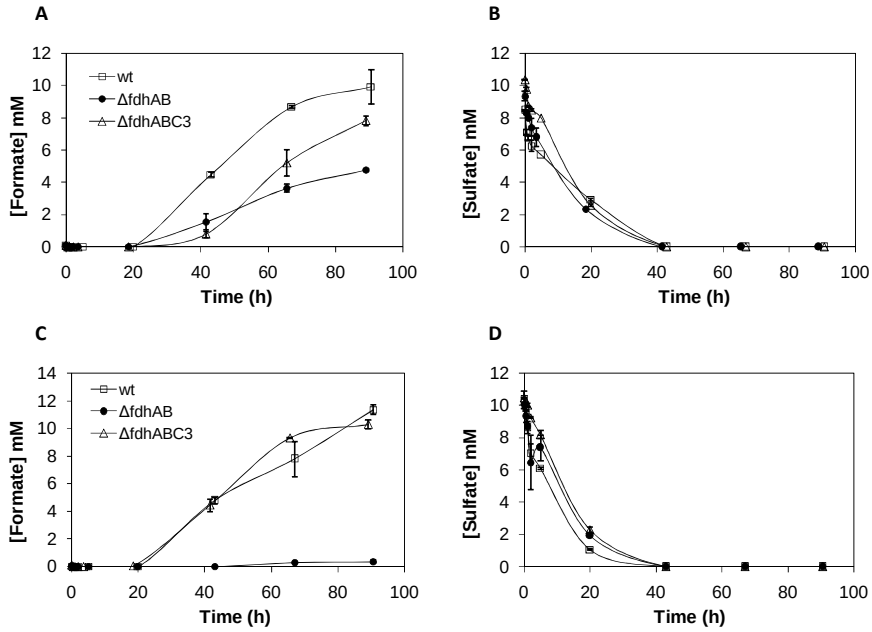

Figure 3.5. Formate production (A, C) and sulfate consumption (B, D) in wt, ΔfdhAB and ΔfdhABC3 cell suspensions incubated with a headspace of hydrogen/CO2 (1bar) and limiting sulfate (10mM), in the presence of Mo (A, B) or W (C, D). The symbols in panels A and C apply to the other panels. Results are means from duplicate experiments ± standard error.

3.5. Discussion

Many bacterial and archeal genomes code for multiple FDHs, and isoenzymes with different functions, expressed under different conditions, with different cell locations or incorporating different metals in the active site often coexist in the same organism [1, 13, 16, 18, 46]. This allows the use of different pathways for energy conservation and adaptation to environmental constrains, like substrate or metal availability. The *D. vulgaris* genome codes for three FDHs, all acting in the periplasmic side of the membrane, which predicts a function mainly as formate dehydrogenases, contributing to the proton motive force and chemiosmotic ATP production when formate is available as growth substrate, or if it is produced by the cell during oxidation of other substrates. The two main enzymes detected are soluble ($FdhABC_3$ and FdhAB) and interact with the cytochrome c pool. In the present work we constructed deletion mutants of each of these enzymes to get insight into their role in energy metabolism during growth with formate, but also lactate and hydrogen, two of the main natural substrates for SRB. In the interpretation of the results it is important to keep in mind that these substrates, as well as the metals Mo and W, are involved in regulating FDH expression [34]. In the presence of Mo the main FDH expressed is $FdhABC_3$, and this is significantly upregulated by formate. In the presence of W the $FdhABC_3$ is down-regulated and FdhAB is the main enzyme, and this is upregulated with both formate and H_2. In addition, $FdhABC_3$ only incorporates Mo in the catalytic

cofactor, whereas FdhAB can incorporate both Mo and W, and FdhAB has a much higher catalytic efficiency than $FdhABC_3$.

With formate as energy source, the role of FDHs is rather straightforward. They oxidize formate releasing protons to create a proton motive force, and electrons which are transferred through membrane-bound carriers to the cytoplasm to reduce sulfate. Upon growth with formate the two mutants revealed different phenotypes in Mo or W. *ΔfdhAB* was the most impaired mutant showing a lower growth rate than *wt* and the *ΔfdhABC*$_3$ mutant in Mo. In the presence of W, the *ΔfdhAB* mutant is unable to grow with formate, due to the down-regulation of $FdhABC_3$ by W and its inability to incorporate this metal. Thus FdhAB is essential for growth in the presence of W, but it also plays a role in Mo conditions, since the *ΔfdhAB* mutant is not identical to the wt. The *ΔfdhABC*$_3$ mutant grows slightly slower than the wt in Mo conditions, but the final biomass yield is considerable lower, which agrees with the fact that $FdhABC_3$ is the main FDH present in formate/Mo conditions [34]. In W the *ΔfdhABC*$_3$ mutant grows at the same rate and with similar cell yield than the wt indicating that $FdhABC_3$ does not really play a role in these conditions, where FdhAB is the main FDH present. Thus, both soluble FDHs play a role during growth with formate

When lactate is used as electron donor for sulfate reduction, substrate level phosphorylation is not sufficient to generate energy, since the same number of ATP molecules are produced through substrate-level phosphorylation as consumed with sulfate activation.

Alternative mechanisms involving the cycling of intermediates, such as hydrogen [47] or CO [37], were suggested as a way to conserve energy. In face of the multiple FDHs encoded in the *D. vulgaris* genome formate cycling (Figure 3.6) was also proposed [31, 36, 37]. Lactate is first oxidized to pyruvate, whose metabolism can proceed through one of three pathways: via pyruvate:ferredoxin oxidoreductase (PFOR), carbon monoxide (CO) dehydrogenase (CODH)/CO-dependent hydrogenase, or via a pyruvate formate-lyase (PFL). PFL produces formate that can be transported across the membrane and oxidized to CO_2 by the periplasmic FDHs. Both mutants showed impaired growth in lactate/sulfate in either Mo or W conditions, and this effect is slightly more pronounced in the *ΔfdhABC*$_3$ mutant. These results show that both FdhAB and FdhABC$_3$ play a role during growth on lactate, and thus provide strong evidence for the formate cycling hypothesis. The growth of the *ΔfdhAB* mutant in W conditions, where both soluble FDHs are suppressed, indicates that either the membrane–associated FDH compensates the absence of the other two, or there is redirection of electrons through the other pathways.

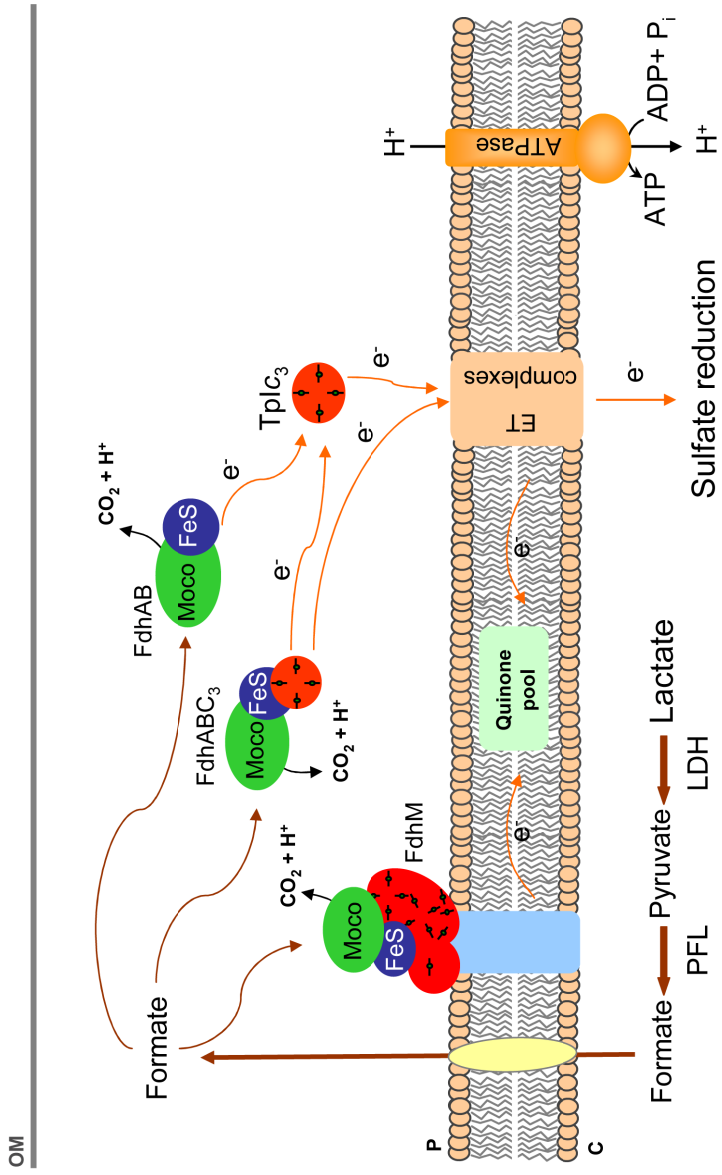

Figure 3.6. Schematic representation of formate cycling during growth of *D. vulgaris* with lactate/sulfate. Formate produced in the cytoplasm by PFL is transported to the periplasm where it is oxidized by FDHs. The resulting electrons and protons are transferred back to the cytoplasm to reduce sulfate and produce ATP, respectively. FdhAB, soluble formate dehydrogenase; $FdhABC_3$, soluble formate dehydrogenase containing a cytochrome c_3-like subunit; FdhM, membrane associated formate dehydrogenase containing two cytochrome *c* subunits; $TpIc_3$, type I cytochrome c_3; PFL, pyruvate formate-lyase; LDH, lactate dehydrogenase.

It was shown previously that in *D. vulgaris* expression of FDHs and PFL activating enzyme is higher when cells are grown with H2 relative to lactate, suggesting that in these conditions FDHs could also be involved in energy conservation [36]. However, the role of FDHs during growth with H_2 is not clear. They may be involved in formate cycling by oxidizing formate produced by PFL in the cytoplasm from pyruvate, generated from CO_2 and acetate. Although acetate needs to be activated in an ATP consuming reaction to form acetyl-CoA, formate production in the cytoplasm and oxidation in the periplasm would contribute to the generation of extra proton motive force and ATP production, in addition to H_2 oxidation. An alternative function for FDHs would be to reduce CO_2 to formate, which could be transported to the cytoplasm and converted to pyruvate, possibly by PFL acting reversibly. In the first hypothesis we would expect formate accumulation in the mutants, since the oxidation rate would be lower than in wt. However, if FDHs are functioning as CO_2-reductases, instead of formate dehydrogenases, then the formate concentration would be lower in the mutants versus *wt*. Either way, growth with H_2 was expected to be affected in both mutants. In contrast, no significant differences in growth were observed between the mutants and the wild-type, indicating that, although formate cycling may be operating when cells grow with H_2, it is certainly not essential. In addition, no formate production was detected during growth.

With concentrated cells suspensions under H_2/CO_2, formate accumulation was observed as sulfate became depleted, and it

accumulated more rapidly and reached higher concentrations in *wt* cells. If PFL was the source of formate, to be reoxidized by the periplasmic FDHs, then the absence of one of the enzymes should lower the rate of formate oxidation making it accumulate in the supernatant at least transiently. Since the mutants accumulate less formate than the wt, this suggests that formate is being produced by the FDHs in wt from CO_2 supplied to medium. Nevertheless, we cannot discard that formate cycling is happening when sulfate is present in the medium and H_2 is the electron donor, despite no formate being detected. When sulfate becomes depleted, and because H_2/CO_2 is still supplied to the cells, excessive reductive power accumulates. In this situation the FDHs function reversely to reduce CO_2, which replaces sulfate as electron acceptor, providing an electron and proton sink for H_2 oxidation. The results indicate that the main FDH acting as CO_2-reductase in these conditions is FdhAB, although in Mo the $FdhABC_3$ also plays a role. Previously, we tested both purified enzymes for CO_2 reduction activity and, surprisingly, we did not detect activity for FdhAB, while a very low activity was detected for $FdhABC_3$ [34]. However, the present results indicate that in vivo, FdhAB functions as CO_2-reductase. W-containing FDHs are proposed to function mainly as CO_2-reductases because of the lower redox potential of W(IV)/W(VI) compared to Mo(IV)/Mo(VI) and many of them are extremely sensitive to oxygen, although *D. gigas* FdhAB appears to be an exception [29, 48]. Most likely the CO_2-

reductase activity of isolated *D. vulgaris* FdhAB was lost upon purification.

The observed production of formate from hydrogen may have biological and environmental significance. Sulfate-reducing bacteria are metabolically versatile, and in the absence of sulfate can also grow in syntrophy. In this case, they ferment organic acids and alcohols, as long as the end-products (acetate, CO_2 and H_2) are consumed by other organisms, keeping their concentration low [49, 50]. Although SRB are generally thought to compete with methanogens for common substrates, in fact they coexist in habitats with low sulfate concentrations, where the sulfate reducers fermentative growth is sustained by the acetate, H_2 and CO_2-consuming methanogens [51, 52]. In syntrophic associations both H_2 and formate are thought to play a role in interspecies electron transfer [2, 6]. These two compounds have similar midpoint redox potentials ($E^{0'}$ H2/H+=-414mV, and $E^{0'}$ formate/CO_2=-432mV), allowing interconversion between the two, and H_2 production from formate has been reported in methanogens [53]. The conversion of formate and H^+ to CO_2 and H_2 ($\Delta G^{0'}$ = -3kJ.mol^{-1}) was not considered to provide enough energy for microbial growth. However, growth on formate was shown to be possible by syntrophic associations of both an acetogen and a methanogen and of a sulfate reducer and a methanogen [54]. More recently, growth of a single organism on formate by production of H_2 was demonstrated with the hyperthermophilic archaeon *Thermococcus onnurineus* NA1, by using

a high formate concentration that resulted in *ΔG* values in the order of -8 to -20kJ.mol^{-1} [55]. Clearly, the interconversion between formate and H_2 can sustain microbial life, depending on the relative concentrations of the two. Given the high affinity of SRB for hydrogen we propose that in certain habitats with high H_2 partial pressures and no sulfate available, these organisms may be able to grow by converting H_2 to formate in syntrophic association with methanogens that consume the formate, but are not as efficient as the SRB in the use of H_2. This would be another expression of the increasingly recognized metabolic flexibility displayed by anaerobic microorganisms, which is particularly apparent in the case of the deltaproteobacterial SRB [20].

In conclusion, this work provided evidence for the occurrence of intracellular formate cycling during growth of *D. vulgaris* Hildenborough with lactate/sulfate, as one of several metabolic pathways the organisms uses to achieve energy conservation in this conversion. Both soluble FDHs play a role in this process. During growth in H_2/CO_2/sulfate the FDHs are apparently not essential, but when sulfate is exhausted they reduce CO_2 to formate, in what may be a newly recognized metabolism that could allow syntrophic growth of SRB in anaerobic environments. Further work will be required to test this hypothesis.

3.6. Acknowledgments

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Chapter 4

Periplasmic electron transfer between formate dehydrogenase and cytochromes *c* in *Desulfovibrio desulfuricans* ATCC27774

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4.1. Summary

Multiple formate dehydrogenase isoenzymes are usually present in *Desulfovibrio* spp., suggesting an essential role in energy metabolism. However, not much is known about the electron transfer pathways involving these enzymes. Electron transfer experiments were carried out to assess potential pathways for electrons resulting from formate oxidation in *Desulfovibrio desulfuricans* ATCC 27774. This organism contains a single soluble periplasmic formate dehydrogenase that includes a cytochrome c_3-like subunit. It has also a unique cytochrome *c* composition, including two cytochromes *c* not yet isolated in other species, the split-Soret and nine-heme cytochromes, besides a TpIc_3 cytochrome. The electron transfer between the formate dehydrogenase and these cytochromes was investigated. The monoheme cytochrome c_{553} is proposed as formate dehydrogenase physiological redox partner. A monoheme cytochrome is predicted in the genome of *D. desulfuricans* ATCC27774, but has not been isolated, so the monoheme cytochrome c_{553} from *D. vulgaris* was used instead. The Dsr complex was also tested as a possible electron acceptor. Formate dehydrogenase was able to reduce the c_{553}, TpIc_3 and split-Soret cytochromes with a high rate. The membrane-associated NhcA and the Dsr complex are also reduced with significant reduction rates, not requiring the involvement of either TpIc_3 or c_{553}. For comparison with formate dehydrogenase, the same experiments were carried out with the [NiFe] hydrogenase from *D. desulfuricans* ATCC27774.

4.2. Introduction

Sulfate-reducing organisms (SRO) comprise a heterogeneous group of prokaryotes found in a wide variety of anaerobic habitats. They play a major role in both the carbon and sulfur cycles in these habitats, since they obtain energy by coupling the oxidation of H_2 or organic compounds to the reduction of sulfate [1, 2].

The sulfate respiratory chain is still one of the least understood anaerobic respiratory processes, and one of its peculiarities is that the terminal reductases responsible for reduction of sulfate are all cytoplasmic and soluble, and thus, do not contribute directly to the creation of proton motive force. Several redox proteins have been isolated from organisms belonging to *Desulfovibrio*, the most studied genus of SRO, which may be implicated in the energy conservation process during sulfate reduction, although the exact mechanism of how this is achieved remains to be fully elucidated. There is evidence to suggest that there is not a single mechanism, but instead several energy conserving pathways are possible, depending on the substrate used as energy source [3]. When hydrogen or formate are used as electron donors the energy conservation mechanism is rather straightforward, since their oxidation releases protons in the periplasm creating a proton gradient across the membrane. The same does not apply to the oxidation of lactate in the cytoplasm, which has to be associated with chemiosmotic energy transduction in order to provide enough energy gain from sulfate reduction. Hence, the cycling of intermediates, such as hydrogen, CO or formate, was

proposed as an alternative mechanism for energy conservation in *D. vulgaris* when growing with lactate as electron donor [4-7]. Whatever the pathway followed, periplasmic *c*-type cytochromes are likely to have a crucial role in the process. SRO belonging to the Deltaproteobacteria class, are distinct from other SRO because of the high content of periplasmic cytochromes *c* [3]. The *c*-type cytochromes are soluble periplasmic proteins, very abundant in *Desulfovibrio* spp., but they may also be associated with membrane-bound complexes [3, 8]. The most widespread *c*-type cytochrome in *Desulfovibrio* spp. is the tetraheme type I cytochrome c_3 (TpIc_3), which is a physiological redox partner of hydrogenases, but also shown to mediate electron transfer between the dimeric formate dehydrogenase (FdhAB) and Qrc, a membrane-bound complex in *D. vulgaris* [9, 10]. The TpIc_3 cytochromes function as intermediates between the periplasmic dehydrogenases, that lack an integral membrane cytochrome *b* subunit for direct quinone reduction and membrane-bound complexes such as Qrc or Tmc/Hmc, which in turn transfer electrons from the periplasm to the menaquinone pool (Qrc) or directly to the cytoplasm in order to reduce sulfate (Tmc) [8, 10-12]. Some hydrogenases and formate dehydrogenases have a dedicated cytochrome c_3 subunit [4, 13, 14]. Extensive biochemical and structural studies have been carried out in order to elucidate the properties and function of TpIc_3, which is reported to act as a proton 'thruster' due to its ability to couple electron and proton transfer, contributing to energy transduction [15]. The cytochrome c_3 family

comprises other members such as the TpIIc_3 cytochrome, a subunit of the Tmc membrane complex [12], the high molecular weight cytochrome (HmcA) and the nine-heme cytochrome (NhcA), also part of membrane-bound complexes [16, 17], or c_3-like subunits of formate dehydrogenase and hydrogenase.

The cytochrome *c* composition varies in different *Desulfovibrio* spp. [3]. *D. desulfuricans* is one of the most studied species of *Desulfovibrio*, and the genome of the *D. desulfuricans* ATCC 27774 strain has recently been made available [18]. Besides sulfate, *D. desulfuricans* can also use nitrate as terminal electron acceptor [19]. However, in the presence of both sulfate and nitrate it still prefers to reduce sulfate, even though the reduction of nitrate is energetically more favourable [20]. Recently, it was shown that the operon coding for nitrate reductase (Nap), responsible for nitrate reduction in the periplasm, is regulated at transcriptional level by nitrate and sulfate[20]. Nap expression is induced by nitrate but it is repressed if the preferred electron acceptor, sulfate, is present even in limited amounts [20].

Three multiheme *c*-type cytochromes were isolated from the soluble fraction of *D. desulfuricans* ATCC27774: the TpIc_3, NhcA, and the split-Soret cytochrome [19, 21]. Apart from the TpIc_3 cytochrome, none of these proteins has been reported yet from other species of SRO. NhcA is a monomeric nine-heme cytochrome *c* that is part of the Nhc membrane redox complex [22]. NhcA is homologous to the C-terminal domain of HmcA, the 16-heme cytochrome c-subunit of

the Hmc complex [16, 23, 24]. Due to the homology of both proteins it was suggested that they have a similar function, which is supported by the fact that the Hmc complex is absent in organisms possessing Nhc [3]. However, the Nhc complex does not have cytoplasmic subunits so it should only transfer electrons between periplasmic proteins and the quinone pool. In contrast, the Hmc complex includes two additional subunits: a membrane-bound cytochrome *b*, and a cytoplasmic subunit of the same family of DsrK and HdrD, the catalytic subunit of heterodisulfide reductases. Recently, a role for Hmc in syntrophic metabolism was proposed, when *D. vulgaris* grows in coculture with a methanogen with lactate as energy source [25]. In *D. desulfuricans* ATCC 27774, NhcA was reported to receive electrons from hydrogenase via TpIc_3, based on a biochemical and structural study of these proteins [24, 26]. However, in another strain, *D. desulfuricans* Essex, it was proposed that NhcA receives electrons directly from hydrogenase [27, 28]. The Split-Soret cytochrome is a dimer of two identical subunits and derives its name from the unusual split observed in the Soret band (420nm, with a shoulder at 415nm) of the ferrycytochrome [29]. Dsr is a strictly conserved membrane complex in SRO and contains a periplasmic three heme cytochrome subunit (DsrJ), unrelated to the cytochrome c_3 family [30]. DsrJ is not reduced by hydrogenase and TpIc_3, and is thought to interact with an unknown periplasmic partner [30].

Although several studies have been carried out concerning electron transfer between hydrogenases, *c*-type cytochromes and

membrane complexes in *Desulfovibrio* spp. (reviewed in [8]), not much is known about the electron pathways involving formate dehydrogenases. Since formate is an important metabolite in natural anaerobic habitats and an important energy source for SRO, it is of interest to understand the routes followed by electrons resulting from formate oxidation. In order to get further insight on the possible electron transfer pathways involved in formate oxidation, we opted to use the organism *D. desulfuricans* ATCC 27774, which has only one soluble periplasmic FDH. We studied the reduction of the cytochromes TpIc_3, split-soret, NhcA, DsrJ and the monoheme cytochrome c_{553}, by FdhABC_3. For comparison, the same set of experiments were performed with the *D. desulfuricans* ATCC 27774 [NiFe]-hydrogenase.

4.3. Materials and methods

4.3.1. Protein purification

The TpIc_3, NhcA and split-Soret cytochromes were purified as described in [19]. FdhABC_3 and [NiFe]-hydrogenase as described in [13, 31], respectively. Dsr was purified as described in [30]. Since cytochrome c_{553} could not be isolated from *D. desulfuricans* we used the protein from *D. vulgaris* in these experiments, which was purified as described in [32].

4.3.2. Enzymatic measurements

Enzymatic assays with formate dehydrogenases and hydrogenases were performed in strict anaerobic conditions inside a glove box. The enzyme activities were first tested with benzyl viologen or methyl viologen, respectively, as described elsewhere [33], prior to their use in the cytochrome reduction experiments. The reduction of cytochromes was measured by following the increase in absorption at 553 nm for cytochrome c_{553} (ε=29.1 $mM^{-1}.cm^{-1}$), 552nm for TpIc_3 (ε=120 $mM^{-1}.cm^{-1}$), split-Soret (ε=120 $mM^{-1}.cm^{-1}$) and NhcA cytochromes (ε=270 $mM^{-1}.cm^{-1}$) and 555nm for the Dsr membrane complex (ε=132.4 $mM^{-1}.cm^{-1}$).

4.3.3. Reductions with *D. desulfuricans* ATCC 27774 formate dehydrogenase

Formate dehydrogenase (purified aerobically) was deoxygenated before all experiments, by leaving the sample for about one hour at 4ºC under the glove box atmosphere, and then diluted to the required concentration in buffer containing 0.2mM DTT. To compare the reduction of the different cytochromes we used the same total heme concentration (15µM). Thus, the concentrations of each cytochrome used depended on the number of heme groups present. The concentrations were: 0.5nM FDH, 15µM c_{553}, 3.75µM TpIc_3 and split-Soret cytochromes, 1.7µM nine-heme cytochrome and 3µM Dsr. All the experiments were repeated at least twice, except with Dsr complex, due to the limited amount available. The

cytochrome solutions were deoxygenated by performing several cycles of argon/vaccum before insertion in the glove box.

4.3.4. Reductions with *D. desulfuricans* ATCC 27774 [NiFe] hydrogenase

The [NiFe] hydrogenase was activated prior all experiments, by performing several cycles of H_2/vaccum followed by incubation under H_2 at 30ºC for one hour. All solutions used in the assays were H_2-saturated. The concentrations of each cytochrome and hydrogenase used were: 6nM [NiFe]-hydrogenase, 15µM c_{553}, 3.75µM TpIc_3 and split-Soret cytochromes, 1.7µM nine-heme cytochrome and 3µM Dsr. All experiments were performed at least twice except with Dsr complex, due to the limited amount.

4.4. Results and discussion

The only FDH reported from *D. desulfuricans* ATCC 27774 is an FdhABC$_3$, a FDH containing a cytochrome c_3-like subunit [13], similar to FdhABC$_3$ from *D. vulgaris* [14]. Analysis of the recently sequenced *D. desulfuricans* ATCC 27774 genome reveals the presence of a second FDH [3] that is facing the periplasm and includes an integral membrane NrfD-type subunit for direct menaquinone reduction. In addition, a cytoplasmic FDH is also present [3]. In *Desulfovibrio* spp. most periplasmic FDHs and hydrogenases are soluble and transfer electrons to *c*-type cytochromes. The presence of multiple FDHs in

Desulfovibrio spp. and other SRO suggests an important role for formate in their energy metabolism. Over the years, several studies have been carried out involving hydrogenases and associated electron transfer pathways [8], but comparatively less is known concerning FDHs. *D. desulfuricans* ATCC 27774 is an interesting model organism for this study, since it contains a single soluble FDH and several cytochromes *c* that could act as potential electron acceptors. In *D. vulgaris*, the monoheme cytochrome c_{553} is proposed to be the FDH physiological redox partner [34]. The gene coding for this cytochrome is found next to the genes for cytochrome *c* oxidases in several several *Desulfovibrio* spp. [35], indicating that c_{553} is the electron donor to the oxidases. Also cytochrome c_{553}:oxidase activity was recently reported in *D. vulgaris* membranes [36, 37]. However, there should also be a pathway to transfer electrons from formate oxidation to sulfate reduction, and in this study several proteins were tested as possible electron carriers in the periplasm. The effect of adding catalytic amounts of TpIc_3 or cytochrome c_{553} as intermediates in the process was also tested.

Prior to the electron transfer experiments FDH activity was tested with formate and an artificial electron acceptor. The specific activity of the purified $FdhABC_3$ (109 $U.mg^{-1}$) is slightly higher than the value previously reported for this enzyme [13]. With isolated $FdhABC_3$, the reduction rates for c_{553} were the highest compared to the other cytochromes (Table 4.1). The presence of catalytic amounts of TpIc_3 had no effect on this reduction rate, when compared to

catalytic amounts of the cytochrome c_{553}. The lack of effect of TpIc_3 is likely due to the fact that this is already present as a subunit of FDH. This subunit was shown to be essential for electron transfer between the enzyme and c_{553} [14, 38].

The reduction rate of TpIc_3 by $FdhABC_3$ was high, and in the same range of the value reported for [NiFe]-hydrogenase and TpIc_3 from *D. vulgaris* [39], but lower than the rate obtained for c_{553} reduction, as previously reported for *D. vulgaris* $FdhABC_3$ [14]. The addition of catalytic amounts of c_{553} caused a small increase in the reduction rate of TpIc_3.

With the split-Soret cytochrome the reduction rate was also quite high, although lower than for TpIc_3, suggesting that this may be a physiologically relevant pathway for electrons resulting of formate oxidation. In this case, the presence of either TpIc_3 or c_{553} did not influence the reduction rate, which means that neither is an intermediate in the reduction. The mRNA and protein levels of split-Soret were shown to be higher in cells grown with nitrate instead of sulfate as electron donor, and the same was reported for $FdhABC_3$ [13, 19, 29]. This suggests that electron transfer from $FdhABC_3$ to nitrate reduction via the split-Soret cytochrome may be a physiological pathway in *D. desulfuricans*. In this organism the dissimilatory nitrate reductase is a periplasmic enzyme [40], whose electron donor is either a membrane-bound NapC cytochrome or a soluble NapM cytochrome [41]. However, neither NapM or NapC have been isolated, so the complete pathway could not be tested.

$FdhABC_3$ was also able to reduce the NhcA at a significant rate, although three-times lower than that obtained with split-Soret cytochrome. Once more, the presence of catalytic amounts of TpIc_3 or c_{553} did not influence NhcA cytochrome reduction rate. As opposed to the split-Soret, NhcA was shown to be present at higher levels when cells grow with sulfate instead of nitrate as electron donor [17].

The lowest reduction rate was obtained for Dsr, suggesting that $FdhABC_3$ is able to transfer electrons directly to the Dsr complex, which in turn transfers them to the cytoplasm for the reduction of sulfate. The [NiFe]-hydrogenase, on the other hand is not able to reduce Dsr, even in the presence of TpIc_3, as reported previously [30], and confirmed in the present work (see below).

Table 4.1. Reduction rates for $FdhABC_3$ from *D. desulfuricans* with each cytochrome, and with or without catalytic amounts of TpIc_3 (from *D. desulfuricans*) or c_{553} (from *D. vulgaris*) (nmol cyt.min^{-1}.nmol $enzyme^{-1}$).

	c_{553}	TpIc_3	split-Soret	NhcA	Dsr
$FdhABC_3$	14062 ± 525	1270 ± 71	710 ± 71	213 ± 13	127
$FdhABC_3/c_{553}$	15505 ± 0	1950 ± 42	686 ± 93	173 ± 19	121
$FdhABC_3$/TpIc_3	15381 ± 175	1160 ± 0	723 ± 85	173 ± 6	88

We also determined the kinetic parameters for the reduction of c_{553}, TpIc_3 and split-Soret by FdhABC$_3$ (Table 4.2). The limited amounts of NhcA and Dsr complex did not allow similar determinations for these two proteins. The highest turnover number and lower K_m were obtained for cytochrome c_{553}. The turnover numbers for TpIc_3 and split-Soret cytochrome were of the same order, whereas the K_m for the split-Soret cytochrome was higher than for TpIc_3.

Table 4.2. Kinetic parameters of FdhABC$_3$ from *D. desulfuricans* with different cytchromes.

	Turnover no. (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}.s^{-1}$)
c_{553}	239	2.7	88
TpIc_3	17.1	3.9	4.4
split-Soret	27	13	2.1

Electron transfer between the [NiFe]-hydrogenase and c_{553}, TpIc_3, split-Soret or NhcA were also tested. The specific activity of [NiFe]-hydrogenase (14 $U.mg^{-1}$), tested prior to the reduction experiments with an artificial electron donor, was 65% below the reported value (40 $U.mg^{-1}$). The attempts to fully reactivate it were not successful, and part of the enzyme remained inactivated. As such,

the absolute reduction values observed are lower than the real ones, but it is still possible to compare the relative reduction rates obtained for each protein. The monoheme cytochrome c_{553} was reduced by the hydrogenase only in the presence of TpIc_3 (Table 4.3). A similar result was obtained for [NiFe]-hydrogenase from *D. vulgaris* and c_{553} [39], although the reduction rate obtained in that case was six-fold lower than the one we obtained here with *D. desulfuricans* hydrogenase. As expected from the low activity of the isolated [NiFe]-hydrogenase, the value obtained for TpIc_3 reduction was five times lower than the one reported for this reaction in *D. vulgaris* [39, 42]. The reduction rate for the split-Soret cytochrome was four times lower than for TpIc_3, and the presence of the last in catalytic amounts did not influence the rate value. The lowest reduction rate was obtained for NhcA, confirming the value previously reported [24], although in the present work we did not observe a catalytic effect of TpIc_3. A similar result was reported for [NiFe]-hydrogenase and NhcA of another strain, *D. desulfuricans* Essex [27].

Table 4.3. Reduction rates for [NiFe] hydrogenase from *D. desulfuricans* with each cytochrome, and with or without catalytic amounts of TpIc_3 (from *D. desulfuricans*) or c_{553} (from *D. vulgaris*) (nmol cyt.min^{-1}.nmol enzyme^{-1}) (means ± standard deviations).

	c_{553}	TpIc_3	split-Soret	NhcA	Dsr
[NiFe] H_2ase	0	320 ± 16	77 ± 5	21 ± 3	0
[NiFe] H_2ase/TpIc_3	2969 ± 107	269 ± 1	85 ± 2	20 ± 0	0

The soluble $FdhABC_3$ from *D. desulfuricans* ATCC27774 includes a cytochrome c_3-like subunit [13]. Thus, the presence of catalytic amounts of TpIc_3 should be redundant, and that explains the fact that very little or no effect at all is visible for this cytochrome in the reduction experiments between $FdhABC_3$ and the other proteins tested. On the other hand, the [NiFe]-hydrogenase does not have a cytochrome c_3 subunit, and TpIc_3 cytochrome, considered as its physiological partner, should be necessary to mediate electron transfer from H_2 oxidation to other soluble or membrane-bound proteins [43]. Nevertheless, in the present experiments the split-Soret and NhcA cytochromes were reduced directly by [NiFe]-hydrogenase, indicating that the TpIc_3 cytochrome is not an absolutely essential intermediate. However, given the very high concentration of TpIc_3 in the cell, it is likely that this always mediates electron transfer from the hydrogenase.

The cytochrome c_{553} was proposed to be FDH physiological partner [34], and it also acts as electron donor for cytochrome *c* oxidase [35-37]. An analysis of the *D. desulfuricans* ATCC 27774 genome shows that cytochrome *c* oxidase is not present in this organism [3]. In addition, the monoheme cytochrome present has no sequence similarity with cytochrome c_{553} from other *Desulfovibrio* spp., suggesting that c_{553} may not be the physiological redox partner of FDH. The c_{553} cytochrome has very high redox potentials (20 to 80 mV), in contrast to the low redox potentials of TpIc_3 (-200 to -400mV). Thus, it is more plausible that TpIc_3 is involved in electron

transfer from formate ($E^{0'}$ formate/CO_2 = -432 mV) to sulfate reduction, rather than c_{553}. Nevertheless, the data obtained in the present work shows that cytochrome c_{553} is indeed an efficient electron acceptor for FDH, but most probably for oxygen, and not sulfate, reduction.

In conclusion, $FdhABC_3$ from D. desulfuricans ATCC 27774 is able to reduce the c_{553}, TpIc_3 and split-Soret cytochromes with a high rate. The membrane-associated NhcA and the Dsr complex are also reduced, although with low rates, not requiring the involvement of either TpIc_3 or c_{553}. This indicates that electrons resulting from formate oxidation may be directly transferred to membrane bound complexes, which in turn transfer them to the menaquinone pool, in the case of Nhc, or to the cytoplasm for sulfate reduction, in the case of Dsr. The fact that periplasmic dehydrogenases can transfer electrons to different acceptors highlights the flexibility of electron transfer pathways operating in *Desulfovibrio* spp.

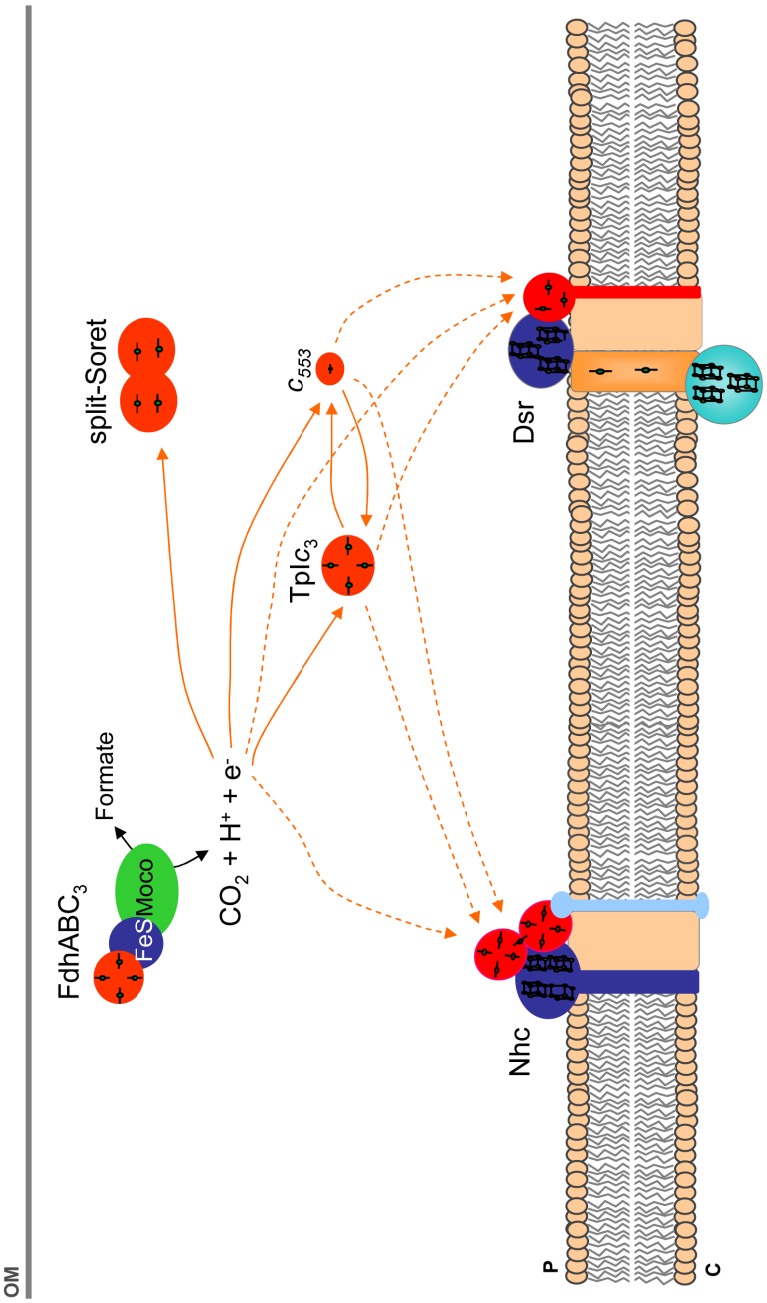

Figure 4.1. Schematic representation of electron transfer between FdhABC$_3$ from *D. desulfuricans* ATCC27774 and several *c*-type cytochromes. FdhABC$_3$, soluble formate dehydrogenase containing a cytochrome c_3-like subunit; TpIc$_3$, type I cytochrome c_3; c_{553}, monoheme cytochrome; Dsr, dissimilatory sulfite reductase membrane complex; Nhc, nine-heme cytochrome *c* membrane complex. Full arrows indicate confirmed electron transfer pathways and dashed arrows possible pathways.

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Chapter 5

A comparative genomic analysis of periplasmic electron transfer pathways in Sulfate Reducing Organisms

The work described in this Chapter was part of the publication:

Inês A. C. Pereira, Ana Raquel Ramos, Fabian Grein, Marta Coimbra Marques, **Sofia M. da Silva**, Sofia Santos Venceslau (2011). *A comparative genomic analysis of energy metabolism in sulfate reducing bacteria and archaea*. Frontiers in Microbial Physiology and Metabolism 2 (69).

Sofia M. da Silva performed the genomic analysis of formate dehydrogenase and periplasmic cytochrome *c* sequences.

5.1. Summary

The number of sequenced genomes of sulfate-reducing organisms (SRO) has increased significantly in the recent years, providing an opportunity for a broader perspective into this type of energy metabolism. In this work we carried out a comparative survey of energy metabolism genes involved in periplasmic electron transfer pathways found in 25 available genomes of SRO, namely of formate dehydrogenases, hydrogenases and cytochromes *c*. The *Deltaproteobacteria* (and *T. yellowstonii*) are characterized by a high number of cytochromes *c*, indicating that periplasmic electron transfer pathways are important in these bacteria. The Archaea and *Clostridia* groups contain practically no cytochromes *c*. However, despite the absence of a periplasmic space, a few extracytoplasmic membrane redox proteins were detected in the Gram-positive bacteria, including a new type of membrane-anchored periplasmic [FeFe] hydrogenase.

5.2. Introduction

Sulfate-reducing organisms (SRO) comprise a phylogenetically diverse group of anaerobic prokaryotes, found ubiquitously in nature, which have in common the ability to grow with sulfate as electron acceptor. As such, they are important players in the global cycles of sulfur and carbon contributing for up to 50% of the total organic matter mineralization in anaerobic marine sediments [1].

The advances made so far in biochemical and genetic studies concerning SRO have allowed the identification of several proteins implicated in their energy metabolism. However, sulfate respiratory chains are not completely understood yet, namely the mechanisms involved in energy conservation. Most biochemical studies have focused on mesophilic sulfate reducers belonging to Deltaproteobacteria, mainly of the *Desulfovibrio* genus, but many other organisms are capable of growing with sulfate and a vast diversity of substrates, such as sugars, aminoacids, methanol or aromatic hydrocarbons, showing the great metabolic diversity present in SRO [2, 3].

The sequencing and comparative analysis of several SRO genomes provides valuable information about possible energetic pathways, and alternative energetic strategies among phylogenetically distant sulfate reducers [4]. The first SRO to have its genome fully sequenced was *Desulfovibrio vulgaris* Hildenborough [5], a well studied strain of *Desulfovibrio* spp. and a model organism for SRO. Since then, the number of sequenced SRO genomes has increased significantly, enabling a broader perspective on the metabolic diversity of these organisms. In the present work a comparative analysis of 25 genomes from SRO, available at the Integrate Microbial Genomes (IMG) website, was performed, focusing on genes/proteins involved in periplasmic electron transfer. The 25 analyzed genomes include: 3 Archaea, 17 Deltaproteobacteria (of the *Desulfovibrionacae, Desulfomicrobiacae, Desulfobacteraceae,*

Desulfohalobiacae, Desulfobulbaceae and *Syntrophobacteraceae* family), 4 *Clostridia* (of the *Peptococcaceae* and *Thermo-anaerobacterales* families) and *Thermodesulfovibrio yellowstonii* DSM 11347 of the Nitrospirae phylum.

Hydrogen and formate are important energy sources for SRO in natural habitats. Oxidation of these substrates by periplasmic enzymes contributes to the generation of a proton gradient as electrons are transferred to the quinone pool or directly across the membrane for cytoplasmic sulfate reduction. When *D.* vulgaris grows with lactate as electron donor, H_2, formate and carbon monoxide, are proposed to be intermediates in a redox cycling mechanism which creates a proton gradient and drives ATP synthesis [6, 7]. The work presented in this thesis (Chapter 3) provided strong evidence that formate cycling is in fact occurring in *D. vulgaris* during growth with lactate. To evaluate if this proposal can be extended to other SRO we carried out an analysis of periplasmic dehydrogenases (hydrogenases and formate dehydrogenases) and cytochromes *c* in the available genomes of SRO. The loci for the analyzed genes can be found in Chapter 8 (Supplementary information) of this thesis.

5.3. Periplasmic formate dehydrogenases

The bacterial uptake hydrogenases and formate dehydrogenases (FDHs) are usually composed of three subunits: a large catalytic subunit, a small electron-transfer subunit and a membrane-associated protein responsible for quinone reduction.

This can either be a NarI-like cytochrome *b* (FdhABC) or a larger protein of the NrfD family (FdhABD). *Desulfovibrio* organisms are unusual in that most of their periplasmic hydrogenases and FDHs lack the membrane subunit, and instead transfer electrons to one or several cytochromes *c* [8]. The soluble periplasmic FDHs can be either, comprise only the catalytic and small subunits (FdhAB) [9] or additionally a dedicated cytochrome c_3 ($FdhABC_3$) [10]. The physiological electron acceptor for FdhAB is also likely to be the soluble Type I cytochrome c_3 (TpIc_3) [11, 12].The soluble FDHs may also transfer electrons directly to membrane-bound proteins such as the Dsr complex or the nine-heme cytochrome (NhcA), as shown in Chapter 4 of this thesis for $FdhABC_3$ from *D. desulfuricans* ATCC27774. These enzymes are also able to function as CO_2-reductases, as reported for *Syntrophobacter fumaroxidans* [13]. In *D. vulgaris* we showed also that both soluble periplasmic FDHs produce formate from H_2/CO_2 in the absence of sulfate (Chapter 3).

Of the SRO analyzed, two Archaea contain neither periplasmic or cytoplasmic FDHs (Table 5.1), indicating that formate metabolism is not essential for sulfate reduction. *Desulfotomaculum acetoxidans* DSM771, member of Clostridia, has only one cytoplasmic FDH, which is involved in formate metabolism during acetyl-CoA pathway, since this organism is a complete oxidizer [14]. *Dm. acetoxidans* is not able to use formate as substrate for growth [14], which is in accordance with the fact that it does not possess a periplasmic FDH. All other

SRO analyzed contain from one to three periplasmic FDHs, the most widespread of which is FdhAB. Six organisms contain one $FdhABC_3$.

Only three organisms contain FdhABC. Interestingly, two Gram-positive bacteria contain FdhABD, where FdhB has a twin-arginine signal peptide, indicating that these enzymes are translocated to outside of the cellular membrane. In *D. vulgaris* Hildenborough the gene locus for FdhABD includes also two cytochromes *c*. Several of the FDHs contain selenocysteine (Sec), and in some organisms only Sec-containing FDHs are present, whereas others contain also Cys-containing enzymes.

Several SRO, mainly Deltaproteobacteria, contain multiple FDHs. These can be differently regulated according to the organism needs. In *D. vulgaris* we showed (Chapter 2) that both soluble periplasmic FDHs are regulated by metal and substrate availability at transcriptional/posttranscriptional level.

5.4. Periplasmic hydrogenases

Two of the SRO analyzed contain no Hases at all: the archaeon *Caldivirga maquilingensis* and the *Deltaproteobacterium Desulfococcus oleovorans*. In addition, *Desulfonatronospira thiodismutans* contains no periplasmic Hases (Table 5.2). The total absence of Hases in two SRO was unexpected and indicates that hydrogen metabolism is not essential for sulfate reduction. The other SRO contain from one to four periplasmic enzymes, the most common of which is the soluble [NiFe] HynAB. All

Deltaproteobacteria contain at least one copy of HynAB. In two archaea and three *Deltaproteobacteria* this protein is membrane-anchored by an additional subunit for quinone reduction (HynABC). Eight organisms also contain the [NiFeSe] HysAB Hase [15]. The HynAB and HysAB enzymes use TpIc_3 as electron acceptor [8]. Finally, only two organisms contain a copy of a HynABC_3, in which another dedicated cytochrome c_3 is encoded next to the *hynAB* genes.

A periplasmic [FeFe] Hase is present in all *Desulfovibrio* organisms, except *D. piger*, and is also found in *S. fumaroxidans*. This enzyme is soluble and also uses TpIc_3 as electron acceptor. A membrane-anchored [FeFe] Hase is present in the four *Clostridial* organisms. A Tat signal peptide present in the catalytic subunit indicates that the enzyme is translocated to the extracytoplasmic side of the cellular membrane, which is somewhat unexpected for the Gram-positive organisms that lack a periplasmic compartment. The enzyme is anchored to the membrane through a NrfD-like transmembrane protein that should transfer electrons to the menaquinone pool.

Overall, the analysis indicates that a periplasmic Hase is found in most SRO, which functions in the uptake of H_2. The *Desulfovibrionacae* organisms contain a higher number of periplasmic enzymes compared to the others. In *D. vulgaris* Hildenborough, which has four periplasmic Hases, it has been shown that expression of these enzymes is fine tuned to respond to metal

availability [16] and hydrogen concentration [17]. The *Clostridial* organisms contain a novel membrane-anchored [FeFe] Hase.

5.5. *c*-type cytochromes

The *Desulfovibrionacae* organisms are characterized by a very high level of multiheme cytochromes *c*, the most abundant and well studied of which is the TpIc_3 [8]. The genome of *D. vulgaris* Hildenborough first revealed that a pool of cytochromes *c* is present in the periplasm [5], some of which belong to the cytochrome c_3 family, but not all [4, 8]. Several multiheme cytochromes *c* are associated with membrane complexes.

Most SRO analyzed contain a high number of multiheme cytochromes *c* (Table 5.3) but several exceptions are observed: *C. maquilingensis*, *Dm. acetoxidans* and *Dm. reducens* contain no cytochromes *c* at all; *Archaeoglobus profundus* contains only DsrJ; *A. fulgidus* contains DsrJ and an octaheme cytochrome, and *Dt. reducens* contains only the NrfHA proteins [18], both with signal peptides again indicating an extra-cytoplasmic location. In general terms, the *Deltaproteobacteria* and *Theromodsulfovibrio yellowstonii* have multiple cytochromes *c,* contrary to the Archaea, Gram-positive SRO and *Ammonifex degensii*. The TpIc_3 is present in all the *Deltaproteobacteria* (except *Desulfotalea psychrophila*) and in *T. yellowstonii*. Often there are 2 to 4 copies of monocistronic cytochromes c_3, whereas others are associated with periplasmic

Hases and FDHs. Tetraheme cytochromes of the c_{554} family [19] are also present in several organisms, including one associated with a methyl-accepting chemotaxis sensory transducer protein, suggesting an involvement in regulation. The split-Soret cytochrome, isolated from *D. desulfuricans* ATCC27774 [20], is present in another six Deltaproteobacteria.

The monoheme cytochrome c_{553} is only present in five *Deltaproteobacteria*, usually in the same locus as cytochrome *c* oxidase, suggesting it acts as its electron donor. In *D. desulfuricans* ATCC27774 genome a monoheme cytochrome is also encoded although it has no sequence similarity with the c_{553} from the other Deltaproteobacteria. This organism doest not possess cytochrome *c* oxidase either. Reduction experiments reported in Chapter 4 showed that FdhABC3 from *D. desulfuricans* ATCC27774 is able to reduce with high rate c_{553} (from *D. vulgaris*), as well as TpIc_3 and split-Soret cytochromes.

The nitrite reductase complex formed by the two cytochromes NrfH and NrfA [18] is one of the more widespread cytochromes in SRO. Nitrite is a powerful inhibitor of SRO and NrfHA acts as a detoxifying enzyme [21].

Table 5.1. Analysis of periplasmic FDH distribution in SRO genomes. N_p, total number of periplasmic FDHs.

		Soluble		Membrane associated	
	N_p	FdhAB	FdhABC3	FdhABC	FdhABD
Archaea					
Archaeoglobus fulgidus	0				
Archaeoglobus profundus	1			1	
Caldivirga maquilingensis	0				
Deltaproteobacteria					
Desulfovibrionacae					
Desulfovibrio aespoeensis	2	2			
Desulfovibrio alaskensis G20	3	3			
Desulfovibrio desulfuricans ATCC 27774	2		1		1
Desulfovibrio magneticus RS-1	3	3			
Desulfovibrio piger	1		1		
Desulfovibrio salexigens	2	1	1		
Desulfovibrio sp. FW1012B	2	2			
Desulfovibrio vulgaris Hildenborough	3	1	1		1
Desulfomicrobiacae					
Desulfomicrobium baculatum	2	2			
Desulfobacteraceae					
Desulfatibacillum alkenivorans	1				1
Desulfobacterium autotrophicum HRM2	3	2	1		
Desulfococcus oleovorans Hxd3	1	1			
Desulfohalobiacae					
Desulfohalobium retbaense DSM 5692	1	1			
Desulfonatronospira thiodismutans ASO3-1	2	2			
Desulfobulbaceae					
Desulfotalea psychrophila	2			2	
Desulfurivibrio alkaliphilus	1			1	
Syntrophobacteraceae					
Syntrophobacter fumaroxidans MPOB	3	3			
Clostridia					
Peptococcaceae					
Desulfotomaculum acetoxidans DSM 771	0				
Desulfotomaculum reducens	1				1
C. *Desulforudis audaxviator* MP104C	1				1
Thermoanaerobacterales					
Ammonifex degensii KC4	1		1		
Nitrospirae					
Thermodesulfovibrio yellowstonii	1	1			
Nr. organisms		13	6	3	5

Table 5.2. Analysis of periplasmic hydrogenases distribution in SRO genomes. N_p, total number of periplasmic hydrogenases.

		Periplasmic [NiFe]				Periplasmic [FeFe]	
		Soluble		Memb		Soluble	Memb
	N_p	HynAB	HysAB	HynABC	HynABC3	HydAB	$[FeFe]_{memb}$
Archaea							
Archaeoglobus fulgidus	1			1			
Archaeoglobus profundus	1			1			
Caldivirga maquilingensis	0						
Deltaproteobacteria							
Desulfovibrionacae							
Desulfovibrio aespoeensis	2	1				1	
Desulfovibrio alaskensis G20	4	1	1		1	1	
Desulfovibrio desulfuricans ATCC 27774	3	1		1		1	
Desulfovibrio magneticus RS-1	3	2				1	
Desulfovibrio piger	2	1	1				
Desulfovibrio salexigens	3	1	1			1	
Desulfovibrio sp. FW1012B	2	1				1	
Desulfovibrio vulgaris Hildenborough	4	1	1		1	1	
Desulfomicrobiacae							
Desulfomicrobium baculatum	2	1	1				
Desulfobacteraceae							
Desulfatibacillum alkenivorans	1	1					
Desulfobacterium autotrophicum HRM2	2	1	1				
Desulfococcus oleovorans Hxd3	0						
Desulfohalobiacae							
Desulfohalobium retbaense DSM 5692	1	1					
Desulfonatronospira thiodismutans ASO3-1	0						
Desulfobulbaceae							
Desulfotalea psychrophila	2	1		1			
Desulfurivibrio alkaliphilus	2	1		1			
Syntrophobacteraceae							
Syntrophobacter fumaroxidans MPOB	2	1				1	
Clostridia							
Peptococcaceae							
Desulfotomaculum acetoxidans DSM 771	1						1
Desulfotomaculum reducens	1						1
C. *Desulforudis audaxviator* MP104C	1						1
Thermoanaerobacterales							
Ammonifex degensii KC4	2		1				1
Nitrospirae							
Thermodesulfovibrio yellowstonii	1		1				
Nr. organisms		15	8	5	2	8	4

Table 5.3. Analysis of distribution of selected cytochromes *c* in SRO genomes. N_t, Total number of multiheme cytochromes *c* detected. The presence of cytochrome *c* oxidases is also indicated.

	N_t	TpIc$_3$	c_{554}-like	split-Soret	NrfHA	c_{553}	Cyt oxid
Archaea							
Archaeoglobus fulgidus	1						
Archaeoglobus profundus	1						
Caldivirga maquilingensis	0						
Deltaproteobacteria							
Desulfovibrionacae							
Desulfovibrio aespoeensis	13	2	1		1		
Desulfovibrio alaskensis G20	14	2	1	1		1	1
Desulfovibrio desulfuricans ATCC 27774	11	1		1	1	1	
Desulfovibrio magneticus RS-1	14	2	3		1	1	1
Desulfovibrio piger	7	1		1	1		
Desulfovibrio salexigens	14	3	1		1		
Desulfovibrio sp. FW1012B	11	2	2		1	1	1
Desulfovibrio vulgaris Hildenborough	18	1	2		1	2	1
Desulfomicrobiacae							
Desulfomicrobium baculatum	15	2	1		1	1	1
Desulfobacteraceae							
Desulfatibacillum alkenivorans	14	2	1				
Desulfobacterium autotrophicum HRM2	15	1	1				1
Desulfococcus oleovorans Hxd3	14	2		1			
Desulfohalobiacae							
Desulfohalobium retbaense DSM 5692	13	4		1			
Desulfonatronospira thiodismutans ASO3-1	11	3	1	1	1		
Desulfobulbaceae							
Desulfotalea psychrophila	5				1		
Desulfurivibrio alkaliphilus	20	2	4	1	1		1
Syntrophobacteraceae							
Syntrophobacter fumaroxidans MPOB	10	1	1		1		
Clostridia							
Peptococcaceae							
Desulfotomaculum acetoxidans DSM 771	0						
Desulfotomaculum reducens	2				1		
C. *Desulforudis audaxviator* MP104C	0						
Thermoanaerobacterales							
Ammonifex degensii KC4	3				1		
Nitrospirae							
Thermodesulfovibrio yellowstonii	10	1	1	1	1		
Nr. organisms		17	13	8	15	6	7

5.6. Conclusions

The comparative genomic analysis reported in this work provides new insights into the energy metabolism of SRO. The intracellular redox cycling of metabolites (like H_2, formate or CO) is not a universal mechanism, but should play a role in bioenergetics of *Deltaproteobacteria* and *T. yellowstonii*, which are characterized by a high number of periplasmic hydrogenases, formate dehydrogenases, cytochromes *c* and cytochrome *c*-associated membrane redox complexes. A high number of cytochromes *c* has previously been correlated with increased respiratory versatility in anaerobes [22], and such versatility is also suggested by the apparent redundancy of periplasmic redox proteins and membrane complexes found in many *Deltaproteobacteria*. Redox cycling is associated with energy conservation through charge separation or redox loop mechanisms. In contrast, the Archaea and *Clostridia* groups contain practically no cytochromes *c* or associated membrane complexes. Despite the absence of a periplasmic space in Gram-positive bacteria, three extracytoplasmic proteins are predicted for these organisms, namely NrfHA, a membrane-anchored FDH and a new type of membrane-anchored periplasmic [FeFe] Hase identified in SRB.

5.7. Acknowledgments

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Chapter 6

Hydrogen as an energy source for the human pathogen *Bilophila wadsworthia*

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6.1. Summary

The gram-negative anaerobic gut bacterium *Bilophila wadsworthia* is the third most common isolate in perforated and gangrenous appendicitis, being also found in a variety of other infections. This organism performs a unique kind of anaerobic respiration in which taurine, a major organic solute in mammals, is used as a source of sulphite that serves as terminal acceptor for the electron transport chain. We show here that molecular hydrogen, one of the major products of fermentative bacteria in the colon, is an excellent growth substrate for *B. wadsworthia*. We have quantified the enzymatic activities associated with the oxidation of H_2, formate and pyruvate for cells obtained in different growth conditions. The cell extracts present high levels of hydrogenase activity, and up to five different hydrogenases can be expressed by this organism. One of the hydrogenases appears to be constitutive, whereas the others show differential expression in different growth conditions. Two of the hydrogenases are soluble and are recognised by antibodies against a [FeFe]-hydrogenase of a sulfate reducing bacterium. One of these hydrogenases is specifically induced during fermentative growth on pyruvate. Another two hydrogenases are membrane-bound and show increased expression in cells grown with hydrogen. Further work should be carried out to reveal whether oxidation of hydrogen contributes to the virulence of *B. wadsworthia*.

6.2. Introduction

A complex and dynamic population of microorganisms colonises the human intestinal tract, and this microflora has a pronounced impact on human physiology [1, 2]. Gut microbial activity has several important consequences for health, namely in recovery of energy, in defence against pathogens, in proper development of the gut, as well as maturation of the immune system [3, 4], by influencing the expression of host genes that participate in fundamental physiological functions. On the other hand, there is evidence suggesting that an inappropriate immune response to colonic bacteria may result in inflammatory bowel diseases [5, 6], and several of these bacteria may behave as opportunistic pathogens, if they can penetrate other tissues, originating infectious processes. The diversity of the gut bacterial community is starting to be unravelled, with the majority of species belonging to novel and uncultured microorganisms [7, 8].

The gut microflora comprises two types of anaerobic bacteria: those that carry out a fermentative metabolism degrading complex polysaccharydes to short-chain fatty acids (mainly butyrate, acetate, propionate and lactate), H_2 and CO_2, and those that are capable of performing anaerobic respiration using these compounds as energy sources [9]. These anaerobic respiratory bacteria include methanogens, sulfate-reducing bacteria, acetogenic bacteria and a few other species including *Bilophila wadsworthia* [10, 11]. *Bilophila wadsworthia* is a strictly anaerobic, gram-negative bacterium first

described in 1989, that comprises a single homogeneous group of organisms [10-12]. This bacterium is an important opportunistic gut pathogen since it is found in a multitude of anaerobic infections, and in particular in appendicitis and intra-abdominal infections [13-15]. It is isolated from about half of appendicitis specimens, being the third most common isolate. *B. wadsworthia* is a member of the normal human faecal flora and has also been found in oral and vaginal fluids, and in environmental samples [11, 16]. It is also present in faeces of other animals, such as pigs, chickens and primates [17-19]. This bacterium performs a very interesting type of respiration in which taurine, one of the major solutes in mammals, is dissimilated with formation of sulphide, acetate and ammonia [16]. *B. wadsworthia* is related to sulfate-reducing bacteria, but does not reduce sulfate [16]. Instead, it adapted to the conditions in the human body, where taurine is abundant, and uses this compound as a source of sulphite, which serves as the terminal electron acceptor for the respiratory chain [16, 20]. Taurine-conjugated bile acids are a likely source of taurine for *B. wadsworthia*, and this organism has also been implicated in bile acid metabolism [21]. Taurine respiration leads to sulphide formation, which is thought to have a role in inflammatory bowel diseases due to its toxic effects on colonic epithelial cells [22-24]. Taurine utilization by *B. wadsworthia* has been well characterised [16, 20, 25, 26] but comparatively less is known about how carbon and energy sources are metabolized by this organism in the colon. It has been reported that it can use several short-chain

fatty acids produced by the fermentative processes, like formate, lactate, pyruvate or others [16]. Another possible energy source in the human colon is hydrogen, a high-energy diffusible reductant also produced by colonic fermentations. It has been shown that the ability to use H_2 as energy source is an important virulence factor for several pathogenic bacteria like *Helicobacter pylori*, *Salmonella enterica* and other enteric bacteria [27, 28]. Hydrogen was shown to be a major energy substrate for *Helicobacter pylori* in the human stomach, [28]. Considering that *B. wadsworthia* is living in a hydrogen rich environment (the human colon), we investigated the possibility that this compound also serves as an efficient energy source for this bacterium. Here, we report that *B. wadsworthia* grows very efficiently with H_2 and that it expresses up to five different hydrogenases in several growth conditions.

6.3. Materials and methods

6.3.1. Cell growth and preparation of crude, soluble and membrane extracts

B. wadsworthia was grown in a base medium containing 19mM NH_4Cl, 17mM NaCl, 2mM $MgCl_2.2H_2O$, 7mM KCl, 0.3mM $CaCl_2.2H_2O$, 1mM K_2HPO_4 and 0.1% of yeast extract. The medium was supplemented with a selenite-tungsten solution, a trace element solution, a vitamin solution, 200µg/l 1,4-naphtoquinone and 2µM

resazurin as in DSMZ medium 503. The pH was brought to 7 with HCl. The medium was supplemented with different electron donors, all at a 40mM concentration, and with taurine as electron acceptor at 10mM. Prior to inoculation a reductant, sodium sulfide hydrate 0.3g/l, was added to the media. The growth conditions tested were: formate/taurine, lactate/taurine, pyruvate/taurine, hydrogen/ acetate/ CO_2/taurine (40mM acetate), pyruvate or taurine (fermentative conditions), and H_2/CO_2 (homoacetogenic growth). All cultures were performed in closed glass flasks containing about two thirds their volume of culture, with the exception of H_2-grown cells that were cultured in a 3L bioreactor, with a flow of H_2/CO_2 (80:20) of 900ml/min and stirring at 250rpm, or homoacetogenic growth conditions that were tested in 30ml glass flasks containing about half their volume of culture (the flasks were laying on their side to maximise the surface area) under an H_2/CO_2 overpressure of 1bar. A 10% inoculum in the same medium to that being tested was used in all cases. Cells were harvested by centrifugation at late log phase and kept at -80ºC. Inside a Coy anaerobic chamber (Ar/H_2 atmosphere) the cells were resuspended in 20mM Tris-HCl buffer pH7.6 containing 1mM ethylenediaminetetraacetic acid (EDTA), 1mM phenylmethanesulfonil fluoride (PMSF), 2mM dithiothreitol (DTT) buffer and DNase. The cells were disrupted by passing twice in a French-press cell under anaerobic conditions. The extract was then centrifuged at 10.000g, 15min, 4ºC to remove cell debris yielding the crude extract, a part of which was ultra-centrifuged at 100.000g,

15min, at 4ºC in order to separate the soluble extract from the membranes. The membranes were resuspended in 20mM Tris-HCl buffer pH7.6, 10% glycerol, 2mM DTT and 2% Triton and centrifuged at 10.000g, 15min, 4ºC to give the membrane extract. The lack of contamination of the membrane extract with soluble proteins was assured by the absence of a peak at 629nm due to the cytoplasmic dissimilatory sulphite reductase. Protein concentrations were determined by the Bradford method with bovine gamma-globulin as standard (BioRad). *Desulfovibrio vulgaris* Hildenborough was grown in lactate/sulfate as described in [29] in the presence of Fe, Ni and Se.

6.3.2. Analytical methods

Acetate and taurine were quantified by HPLC using an Aminex HPX-87H (300x7.8mm) column (BioRad) at 60ºC, with 0.005N H_2SO_4 as isocratic mobile phase (0.5ml.min^{-1}) and a Refractive Index detector, LKB2142. Ammonia quantification was carried out by the Berthelot colorimetric method as described [30].

6.3.3. Protein purification

The soluble extract of *B. wadsworthia* cells grown in lactate/taurine was prepared as described above to be used for purification. All chromatographic steps were performed at 4ºC. *Step 1.* The soluble fraction was first purified on a Q-Sepharose High Performance 26/10 (Pharmacia) column equilibrated with 20mM Tris-HCl buffer pH7.6, 2mM DTT and 1mM PMSF. A step gradient with

100mM Tris-HCl buffer pH7.6, 2mM DTT, 1mM PMSF and 1M sodium chloride was performed. *Step 2.* The fractions with hydrogenase and formate dehydrogenase activity were concentrated and further purified on a Q-Sepharose High Performance 16/10 (Pharmacia) column. The buffers were the same as used on the first step. *Step 3.* The fractions with hydrogenase and formate dehydrogenase activity from the second column were again purified on a Mono Q HR 5/5 (Pharmacia) column equilibrated with 20mM Tris-HCl buffer pH7.6 and 2mM DTT. This buffer was used to wash the column before starting a step gradient with 100mM Tris-HCl buffer pH7.6, 2mM DTT and 1M sodium chloride.

6.3.4. Enzymatic activities

Enzymatic activities were determined inside the anaerobic chamber, except for the hydrogen production and formate-hydrogen lyase activities. All assays were performed in 1ml total volume. All the buffers were Ar-saturated with the exception of the one used in hydrogenase activity, which was H_2-saturated. Formate dehydrogenase activity was assayed in 50mM Tris-HCl buffer pH7.6, with 2mM benzyl viologen and 10mM DTT. The reaction was started by adding substrate (20mM sodium formate) and was followed measuring absorption at 555 nm. Lactate dehydrogenase activity was assayed in 50mM phosphate buffer pH 7.8 with 0.08mM 2,6-dichlorophenolindophenol sodium salt. The reaction was started by adding 10mM sodium lactate 50% (w/v) and was followed measuring

absorption at 600nm. Pyruvate oxidoreductase activity was assayed in 50mM Tris-HCl buffer pH8.5 with 0.1mM Coenzyme A, 1mM methyl viologen (electron acceptor) and 10mM DTT. The reaction was started by adding 10mM sodium pyruvate and was followed measuring absorption at 604 nm. Pyruvate-formate lyase activity was assayed as described [31] in 100mM potassium phosphate buffer pH7.4 with 0.08mM Coenzyme A, 1mM Nicotinamide Adenine Dinucleotide (NAD), 6mM sodium-DL-malate, 2mM DTT, 1.1U/ml of citrate synthase and 22U/ml of malate dehydrogenase. The reaction was started by adding 20mM sodium pyruvate and was followed measuring absorption at 340nm. The samples were previously activated by incubation at 37ºC for 5min in a mixture as described [32]. Hydrogenase activity (consumption of H_2) was assayed in H_2-saturated 50mM Tris-HCl buffer pH 7.6 using 2mM methyl viologen as electron acceptor. The reaction was started by adding the sample and was followed measuring absorption at 604nm. Hydrogen production activity of purified fractions was assayed in 50mM Tris-HCl buffer pH7.6 with 1mM methyl viologen and 15mM of dithionite as electron donors. Formate-hydrogen lyase activity was assayed in 50mM Tris-HCl buffer pH7.6 with 20mM sodium formate and 0,0001% resazurin. Quantification of hydrogen in the gaseous phase for hydrogenase and formate hydrogen lyase activities was carried out by Gas Chromatography as described [29].

6.3.5. Activity-stained gels

For activity-staining samples were run in a 7.5% polyacrilamide gel containing 0.1% Triton X-100 in non-denaturing conditions. The running buffer also contained 0.1% Triton X-100. All these procedures were performed inside the anaerobic chamber. The gel for hydrogen activity staining was then placed in a closed flask with degassed 50mM Tris-HCl buffer pH8, 0.5mM methyl viologen, and flushed with H_2 gas. The gel for formate dehydrogenase activity was also placed in a closed flask with degassed 100mM phosphate buffer pH7.4, 1mM methyl viologen, 5mM sodium dithionite, 20mM sodium formate and flushed with argon. The bands were fixed by adding a 10mM 2,3,5-triphenyltetrazolium chloride solution.

6.3.6. Western blot analysis

The samples were run in a 7.5% native gel as described above. Proteins were then transferred to 0.45µm PVDF membranes (Roche) for 1h at 100mV and 4ºC, in a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad). The membranes were equilibrated with Tris-buffered saline solution with Tween 20 (TBST) (20mM Tris-HCl pH7.5, 150mM NaCl, 0.05% Tween 20), and then treated with antiserum raised against the [FeFe] and $[NiFe]_1$ hydrogenases of *D.vulgaris* Hildenborough [29]. The [FeFe] and $[NiFe]_1$ hydrogenases antisera were diluted 1:5000 in TBS (Tris-buffered saline solution without Tween 20). Unbound antibodies were removed by three 5 min washes with TBST. Immunodetection of bound antibodies was done

by treatment with anti-mouse immunoglobulin G (H+L) alkaline phosphatase conjugate (Promega), diluted 1:5000, followed by a solution of nitro blue tetrazolium salt and 5-bromo-4-chloro-3-indolyl phosphate toluidine salt (NBT/BCIP solution from Roche).

6.4. Results and discussion

6.4.1. Growth experiments

B. wadsworthia is usually grown with formate as carbon and energy source. For a preliminary characterization of how other possible energy sources are metabolized by this organism, we compared growth in the standard formate/taurine conditions to growth with alternative electron donors, namely hydrogen (acetate/CO_2 as carbon source), lactate and pyruvate, using taurine at similar concentration as electron acceptor. For comparison we tested also fermentative growth with pyruvate or taurine, and homoacetogenic growth in the absence of taurine. Bacterial growth was followed by optical density measurements and cells were harvested at the end of the exponential growth (Figure 6.1A and Table 6.1). The highest growth rate was observed in H_2/taurine (H_2/T) medium. The growth rate for the standard formate/taurine (F/T) medium was lower in comparison to that in H_2/T and there was a longer lag phase before exponential growth began. A similar lag phase was observed in lactate/taurine (L/T), and this was more

pronounced in pyruvate/taurine (P/T), suggesting a longer adaptation of the cells to the latter growth conditions. The growth rate with L/T was slower than with F/T, and this difference was even more pronounced for the case of P/T. Fermentative growth with pyruvate (P) or taurine (T) was observed, but was very slow in comparison to other conditions. Fermentative growth of *B. wadsworthia* in pyruvate had been previously documented [16], and we show here that this organism has also the ability to ferment taurine (Figure 6.1 and Table 6.1). Taurine fermentation has only been described for two other other organisms [33, 34]. No growth was observed in H_2/CO_2 in the absence of acetate and taurine, which indicates that *B. wadsworthia* is not capable of homoacetogenic growth (results not shown). Overall, these results indicate clearly that hydrogen is in fact a very good energy source for *B. wadsworthia* from which it sustains a rapid and efficient growth. Consumption of taurine during growth with hydrogen was accompanied by formation of acetate and ammonia (Figure 6.1B). Sulphide was not quantified as hydrogen sulphide is removed from the medium by the continuous flow of H_2/CO_2 gas.

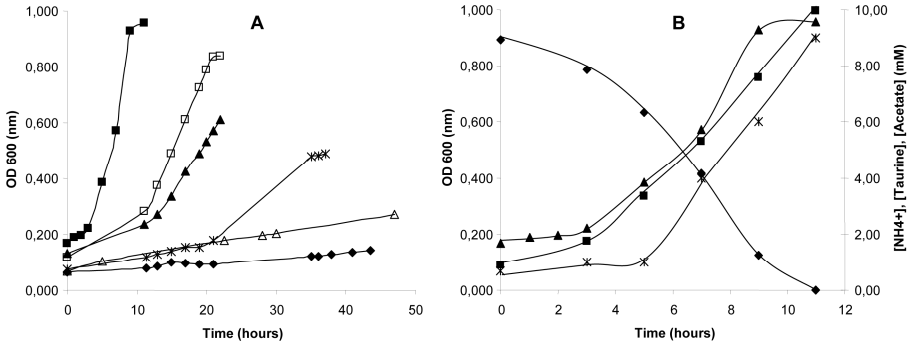

Figure 6.1. A) *B. wadsworthia* growth curves. ■ H_2/taurine; □ formate/taurine; ▲ lactate/taurine; * pyruvate/taurine; Δ taurine; ♦ pyruvate; (representative results from two experiments). **B)** Product formation and subtrate consumption during growth in H_2/taurine. ▲ OD_{600}; ■ Concentration of ammonia produced; ♦ Concentration of taurine consumed; * Concentration of acetate produced.

Table 6.1. Growth of *B. wadsworthia* with different substrates (representative results from two experiments).

Medium	Time (h)	$OD_{600\ nm}$	Biomass yield (g/l)
H2/Taurine	11	0.959	1.07
Formate/Taurine	22	0.838	1.07
Lactate/Taurine	22	0.610	0.85
Pyruvate/Taurine	37	0.490	0.71
Taurine	47	0.272	0.36
Pyruvate	43	0.144	0.19

6.4.2. Quantification of hydrogenase, formate dehydrogenase and pyruvate oxidoreductase activities in cell extracts

We next investigated the presence of hydrogenases in *B. wadsworthia*, and for comparison tested also other main dehydrogenases, as well as lyases, involved in oxidation of the energy sources used. Thus, we tested the enzymatic activities of hydrogenase, formate dehydrogenase, lactate dehydrogenase, pyruvate oxidoreductase, formate hydrogen-lyase and pyruvate formate-lyase in soluble and membrane extracts of cells obtained in most of the conditions described above. All these enzymes are very sensitive to oxygen inactivation, and we confirmed that the use of strict anaerobic conditions, both for preparation of the cell extracts as well as determination of activities, was essential to obtain high and reproducible values for these activities. Thus, all the work was performed inside an anaerobic chamber, except for cell breakage in the French press or centrifugation, steps which were also carried out in strict anaerobic conditions. Despite these precautions the values of lactate dehydrogenase measured were extremely low and unreliable, and so are not reported. Also, no activity could be detected for formate hydrogen lyase or pyruvate formate lyase, which are enzymes also known to be highly sensitivity after cell breakage.

Hydrogenases carry out the reversible oxidation of molecular hydrogen, being able to catalyze either hydrogen uptake or evolution depending on the energy state of the cells. These functions are often associated with different enzymes and cellular localizations, with

hydrogen evolution being most often cytosolic and hydrogen uptake usually periplasmic or membrane-associated [35]. Some bacteria contain two or more different hydrogenases localized in different cell compartments, which reflects the importance of hydrogen in their metabolism. The *B. wadsworthia* cell extracts displayed quite high levels of H_2-uptake hydrogenase activity (Figure 6.2, panel A). This activity was detected in both soluble and membrane extracts, suggesting the presence of more than one hydrogenase. The level of activity was higher in the membrane compared to the soluble fraction for all growth conditions, with the exception of fermentative growth on pyruvate where the opposite is observed. This indicates that fermentative growth may induce the expression of a soluble hydrogenase that may be responsible for producing H_2 as a product of fermentation. In F/T grown cells the level of hydrogenase activity in the soluble fraction is lower than in all other conditions. The total hydrogenase activity was highest for cells grown in hydrogen, where the increase observed was specifically in the membrane fraction. This indicates that in *B. wadsworthia* the hydrogenase responsible for H_2 oxidation during growth on this substrate is membrane-bound, as observed for many other bacteria [36]. For comparison we determined the hydrogenase activity of cell extracts of *Desulfovibrio vulgaris* Hildenborough, a model organism for sulfate-reducing bacteria whose genome encodes for six different hydrogenases [37]. This organism is known to contain high levels of hydrogenase activity and the levels determined for standard lactate/sulfate growth

conditions are in fact quite similar to the levels observed for *B. wadsworthia* grown in H_2/T. This confirms that *B. wadsworthia* is capable of expressing hydrogenase(s) with a high level of activity.

Formate is the standard substrate used to grow *B. wadsworthia* so the activity of formate dehydrogenase was also measured. Formate dehydrogenases are a diverse group of enzymes that catalyze the oxidation of formate to CO_2 and H^+ [38, 39]. For many anaerobes, formate formed from pyruvate is the main electron donor for the respiratory chain. The formate dehydrogenases from these organisms contain either molybdenum or tungsten bound to a pterin cofactor, as well as other redox centres, and are usually very oxygen-sensitive. Several formate dehydrogenases may be expressed in a single organism, as has been shown for *E. coli* where three formate dehydrogenases are expressed in differential conditions [40]. The *B. wadsworthia* cell extracts had high levels of formate dehydrogenase activity (Figure 6.2, panel B), which were considerably higher than those observed for *D. vulgaris*. The highest levels of activity were found in the P/T and P grown cells, and not in the F/T grown cells. The cells grown with H_2/T displayed only a slightly higher level of formate dehydrogenase activity than the F/T or L/T cells, which were similar. The formate dehydrogenase activity was found in both soluble and membrane extracts for all growth conditions. In F/T grown cells the activity was higher in the membrane fraction, whereas in fermentative growth the great majority of the activity was in the soluble fraction. For the three other conditions the formate

dehydrogenase activity was almost similar in both cellular extracts. These results suggest that *B. wadsworthia* may express at least two different formate dehydrogenases, one soluble and another localized in the membrane. As in other bacteria, the membrane-bound formate dehydrogenase is probably responsible for transferring electrons to the respiratory chain, whereas a soluble formate dehydrogenase may be involved in fermentative growth, like the *E. coli* Fdh-H that is part of the formate-hydrogen lyase complex expressed in fermentative conditions [40]. However, we could detect no activity of formate hydrogen-lyase in *B. wadsworthia* soluble extracts, but this may have been due to the high lability of this protein complex.

Another important enzyme in energy metabolism of anaerobes is the Pyruvate:ferredoxin oxidoreductase, which has a crucial role in catalyzing the reversible oxidation of pyruvate by ferredoxin to acetyl-CoA and CO_2 [41]. The pyruvate:ferredoxin oxidoreductase activity of *B. wadsworthia* cells grown in F/T or H_2/T was very low, which agrees with the fact that pyruvate is not necessarily involved in these two metabolic conditions (Figure 6.2, panel C). Growth in the three other conditions induced expression of the pyruvate:ferredoxin oxidoreductase with the highest activity being obtained for P/T grown cells. As in other bacteria, this enzymatic activity is essentially found in the soluble fraction.

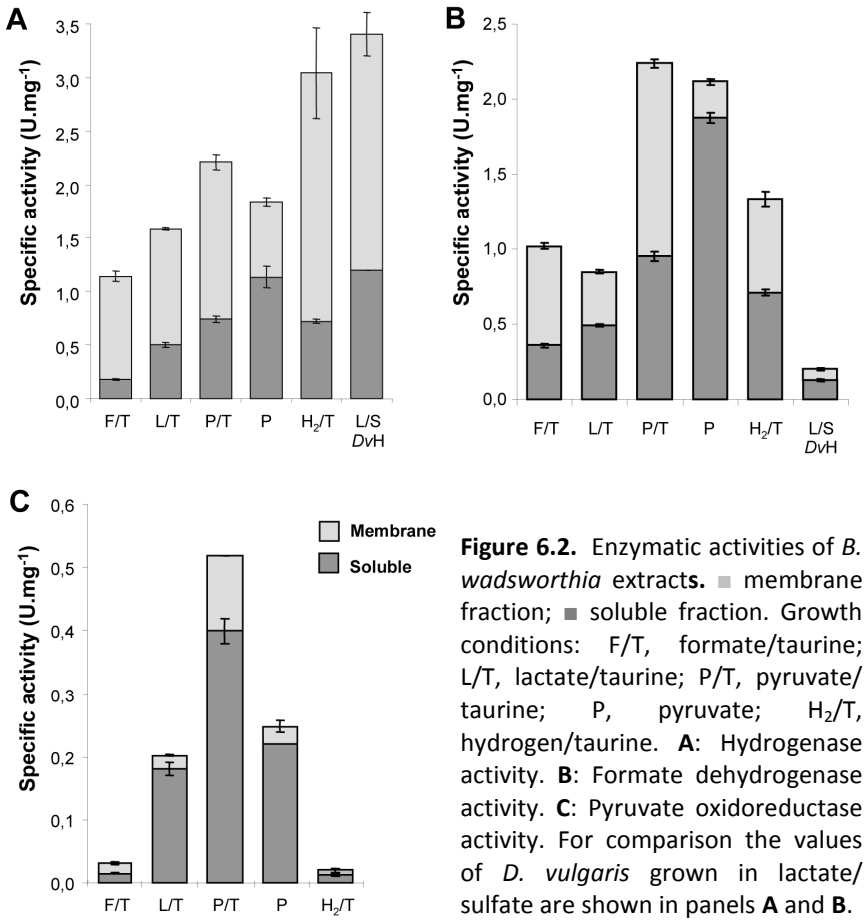

Figure 6.2. Enzymatic activities of *B. wadsworthia* extract**s.** ■ membrane fraction; ■ soluble fraction. Growth conditions: F/T, formate/taurine; L/T, lactate/taurine; P/T, pyruvate/ taurine; P, pyruvate; H_2/T, hydrogen/taurine. **A**: Hydrogenase activity. **B**: Formate dehydrogenase activity. **C**: Pyruvate oxidoreductase activity. For comparison the values of *D. vulgaris* grown in lactate/ sulfate are shown in panels **A** and **B**.

6.4.3. Detection of hydrogenase and formate dehydrogenase isoenzymes

To try to identify the number of isoenzymes of hydrogenases and formate dehydrogenases present in *B. wadsworthia*, and their relative expression, we ran native gels that were stained for hydrogenase and formate dehydrogenase activities [42]. In hydrogenase-stained gels four bands are observed and labelled 1, 2,

3 and 4 (Figure 6.3, A and B). In F/T only one hydrogenase, labelled 2 (Hyd2) is detected in the soluble extract, whereas the four hydrogenases can be detected in the membrane extract. Hyd2 is the only hydrogenase that appears to be constitutive. In P/T and L/T media two hydrogenases show increased expression relative to F/T, Hyd1 and Hyd3, particularly in the membrane extract. In fermentative conditions with pyruvate one hydrogenase, Hyd4, shows increased expression that is detected in the soluble extract. In cells grown with H_2/T the hydrogenases Hyd1 and Hyd3 also show increased expression, mainly in the membrane extract, and Hyd4 is also more prominent in the soluble extract than in F/T conditions.

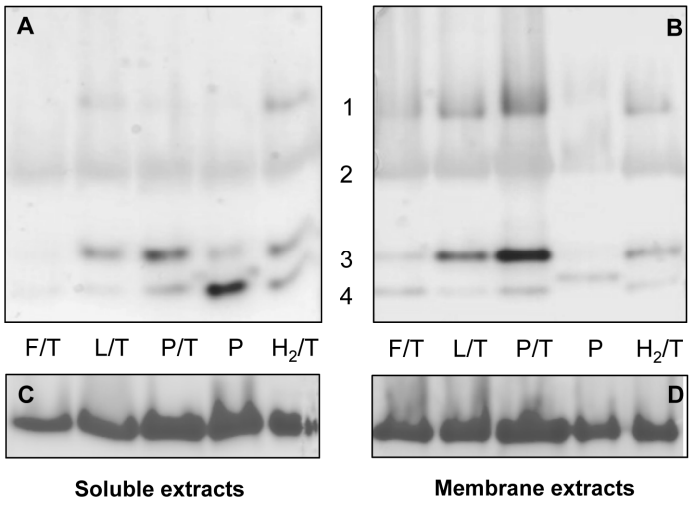

Figure 6.3. Native gels of *B. wadsworthia* cell extracts. Gels **A** and **B** - stained for hydrogenase activity; Gels **C** and **D** - stained for formate dehydrogenase activity; Growth conditions: F/T, formate/taurine; L/T, lactate/taurine; P/T, pyruvate/taurine; P, pyruvate; H_2/T, hydrogen/taurine. Gels **A** and **C** - soluble extracts, Gels **B** and **D** - membrane extracts.

Given the similarity of *B. wadsworthia* to sulfate-reducing bacteria, we tested whether any of the hydrogenases present in the extracts of P/T and H_2/T grown cells, which showed the highest specific activities, could be recognised with antibodies against the [FeFe] and $[NiFe]_1$ hydrogenases from *D. vulgaris* Hildenborough [29]. The vast majority of hydrogenases belong to the groups of [NiFe] and [FeFe] hydrogenases, two phylogenetically distinct classes of enzymes [36, 43]. No hydrogenases reacted with antiserum against the $[NiFe]_1$ hydrogenase (results not shown). In contrast, the two bands corresponding to Hyd3 and Hyd4 in the soluble extracts reacted with the antiserum against the [FeFe]-hydrogenase (Figure 6.4). Interestingly, the strong band present in the membrane extract of P/T cells at the position labelled Hyd3 was not recognised by the anti-[FeFe] antibodies. Because it is membrane associated and it shows increased expression during growth with H_2/T, this hydrogenase is most likely an uptake [NiFe] hydrogenase that is sufficiently different from the *D. vulgaris* $[NiFe]_1$ hydrogenase so that it is not recognised by the antibodies. These results indicate that two hydrogenases of *B.*

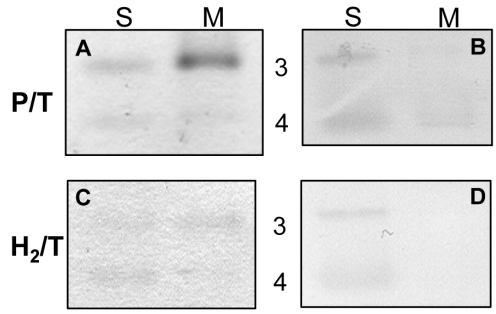

Figure 6.4. Hydrogenase activity stained native gels and Western blots of *B. wadsworthia* P/T and H_2/T cell extracts. A and C: activity-stained gels; B and D: Western blots using antibodies against the [FeFe] hydrogenase of *D. vulgaris*. S=soluble extracts; M=membrane extracts.

wadsworthia run in a similar position (corresponding to Hyd3) in the native gel, but one is a soluble [FeFe]-hydrogenase ($Hyd3_{sol}$) and the other is a membrane-bound, probably [NiFe]-hydrogenase ($Hyd3_{mem}$).

Altogether, these results indicate the presence of five different hydrogenases in *B. wadsworthia*. Hyd2 is constitutive and is detected in both soluble and membrane extracts, which confounds its cellular location. It may be a soluble protein that interacts strongly with membrane-associated proteins or a peripherally-associated membrane protein. The hydrogenases Hyd1, $Hyd3_{mem}$ and $Hyd3_{sol}$ show increased expression with growth in L/T, P/T and H_2/T. Hyd1 and $Hyd3_{mem}$ are membrane-associated, even though a small amount of Hyd1 can also be detected in the soluble fraction, particularly of cells grown with H_2. A similar behaviour was observed for the membrane-bound [NiFeSe]-hydrogenase of *D. vulgaris* grown with H_2/sulfate [29], and it may reflect the strong expression of the H_2-uptake hydrogenase (the $Hyd3_{mem}$ for *B. wadsworthia* and the [NiFeSe] hydrogenase for *D. vulgaris*) in these growth conditions. The $Hyd3_{sol}$ and Hyd4 are both soluble [FeFe]-hydrogenases. Hyd4 is the main hydrogenase present in fermentative conditions.

In contrast to the multiple hydrogenases, the formate dehydrogenase-stained gels revealed a single band for every growth condition in both soluble and membrane extracts (Figure 6.3, C and D). The band is identical in both extracts, which suggests that only one enzyme may be present. Most likely this is a soluble enzyme that interacts with membrane proteins, as described above, and thus is

also detected in the membrane fraction. The relative intensity of the band is higher in pyruvate-containing media, which agrees with the results obtained for the specific activities.

6.4.4. Partial purification

We did a partial purification of the *B. wadsworthia* soluble extract of cells grown in lactate/taurine medium, to try to isolate some of the hydrogenases and the formate dehydrogenase. The purification steps were not performed anaerobically, but DTT was included in all buffers in an attempt to prevent O_2 damage to the enzymes. Three purification steps were performed following hydrogenase and formate dehydrogenase activity. However, we were not able to achieve full purification of the enzymes due to limited amount of cell material, since it is not possible to grow *B. wadsworthia* in large volumes for safety reasons (Class II microorganism). For the formate dehydrogenase two peaks were separated in the first column, but these showed identical bands in the activity-stained native gel, so they may correspond to the same protein. However, it is not possible to discard the possibility that two formate dehydrogenases are present that run in the same position in the native gel. Probably due to oxygen inactivation, the amount of activity recovered after the third column was too low to enable further characterisation. For the hydrogenases it was possible to separate the $Hyd3_{sol}$ and Hyd4 hydrogenases after three purification steps. The other hydrogenases were not detected during the

purification. As for the formate dehydrogenase we observed a strong loss of activity probably due to oxygen damage. Using Western blot we confirmed that the two hydrogenases belong to the family of [FeFe] hydrogenases (Figure 6.5), as indicated from analysis of the cell extracts.

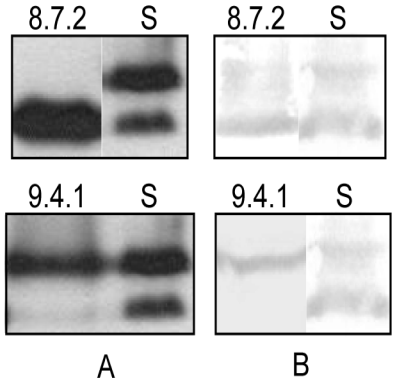

Figure 6.5. Hydrogenase activity-stained native gels (A) and Western blots using antibodies against the *D. vulgaris* [FeFe] hydrogenase (B) of two fractions obtained from purification (fractions 8.7.2 and 9.4.1). S = soluble fraction.

6.5. Conclusions

This work shows that the bile-resistant, human pathogen *B. wadsworthia* can efficiently utilize molecular hydrogen as energy source for growth. This property is likely to be a virulence factor for this organism as it has been shown for other pathogenic bacteria. Up to five different hydrogenases can be expressed by *B. wadsworhtia*, two of which are soluble and belong to the group of [FeFe] hydrogenases. Another two are membrane-associated, of which one is likely to be a [NiFe] uptake hydrogenase. One hydrogenase is expressed in all growth conditions revealing the importance of hydrogen in the metabolism of *B. wadsworthia*, and one of the

soluble [FeFe] hydrogenases is specifically induced in fermentative conditions. Further work will have to be carried out to assess whether hydrogen oxidation contributes to the pathogenicity of *B. wadsworthia*.

6.6. Acknowledgments

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Chapter 7

Concluding remarks

Although a lot of studies have been dedicated to sulfate reducers since their discovery, reflecting their importance in the global cycling of carbon and sulfur, several aspects remain to be fully elucidated, specially concerning the way energy conservation is achieved in this phylogenetically diverse group of microorganisms. This group is known for their highly flexible energy metabolism, which is expressed in multiple metabolic pathways within each organism. This is particularly striking in the deltaproteobacterial SRB, as observed in our genome analysis. These organisms contain multiple isoenzymes of hydrogenases, formate dehydrogenases, cytochromes *c* and transmembrane redox complexes, suggesting that intracellular redox cycling of intermediates such as H_2 and formate is a relevant process in the energy metabolism, which is likely to contribute to their fitness and resilience. We also observed this flexibility in terms of possible electron transfer pathways in the periplasm. In *Desulfovibrio desulfuricans* periplasmic dehydrogenases are able to transfer electrons to several cytochromes *c*, providing different pathways for electrons to be carried from the periplasm to the cytoplasm, where sulfate reduction occurs. Metabolic flexibility is an advantageous trait since it allows the organisms to adapt and thrive in changing and sometimes adverse environmental conditions.

Sulfate reducers have a sulfidogenic metabolism in the presence of sulfate, but in its absence they are able to live in syntrophy with methanogens or syntrophs. Hydrogen is known for a long time as an important metabolite in syntrophic communities, and

several studies have been dedicated to its metabolism in anaerobic habitats as well as to its main enzyme, hydrogenase. Formate is also an essential metabolite in anaerobic habitats not only as source of energy and/or carbon, but also as an intermediate in several pathways and as an electron transfer metabolite for organisms living in syntrophy. Formate dehydrogenase is the main enzyme involved in formate conversion and, although formate is energetically equivalent to H_2, with a similar redox potential, much less is known about its metabolism.

The importance of formate for SRB is depicted in their genomes, which can code for multiple formate dehydrogenases. In the present work we studied the metabolism of formate, and its key enzyme formate dehydrogenase, in sulfate reducers belonging to *Desulfovibrio* spp.. *Desulfovibrio vulgaris* genome codes for three periplasmic FDHs, two soluble and one membrane associated. By generating mutants for each soluble FDH we were able to determine the effects in metabolism caused by the absence of these enzymes. The results obtained provided strong evidence for the formate cycling hypothesis as an energy conserving mechanism during growth of *D. vulgaris* on lactate/sulfate. We also showed that formate dehydrogenases in *D. vulgaris* function as CO_2 reductases in the absence of sulfate and with H_2 as electron donor. This may provide for a temporary electron and H_2 sink, which may be advantageous in natural conditions where H_2 concentration does not remain stable throughout time. Formate would be produced when H_2

concentration is high, as a soluble way of storing reducing power, which could be used directly or reconverted to H_2 when its concentration was lower. Formate, or H_2 resulting from formate conversion, could also be used by a syntrophic partner like a methanogen. Further work is being carried out in order to determine if this process of formate production may contribute to energy conservation. If this is confirmed, then it will constitute yet another alternative mode for energy conservation in SRB. In the future it would also be interesting to create a third mutant for the membrane associated FDH, to determine its role in metabolism and understand how this role integrates with what we learned about the two soluble FDHs in the present work. Another interesting experiment would be to generate double (and triple) mutants and try to discriminate between specific functions of each FDH in energy metabolism.

Formate dehydrogenase activity depends on the presence of a metal, molybdenum or tungsten, coordinated to an organic cofactor, pyranopterin. It is one of the few enzymes able to incorporate either Mo or W in its active site. Concerning *D. vulgaris*, we used several approaches such as protein purification, Western-Blot, real-time qPCR and FDH activity-stained gels to show for the first time that formate dehydrogenases are regulated at transcriptional/ posttranscriptional level by Mo and W. The soluble, two subunit formate dehydrogenase, FdhAB, was shown to be able to incorporate both metals in its active site, while $FdhABC_3$ showed a high selectivity for Mo. We could not conclude on how the presence of either Mo or

W in the active site of FdhAB influences its specific activity. This should be a topic to assess in the future in order to determine if the higher catalytic efficiency of this enzyme is indeed due to the presence of W, which has a lower redox potential than Mo. Not much is known about Mo and W regulation and how each of these metals is selectively incorporated in the enzymes active site. However, the selectivity in metal incorporation and the ability to regulate FDH expression according to the metal levels will certainly constitute an advantage for anaerobic organisms such as *D. vulgaris,* which live in environments with variable Mo and W concentrations.

Some *Desulfovibrio* species and other members of the Desulfovibrionaceae family are part of the normal human intestinal flora, but in several situations they may spread to other tissues and become pathogens. *Bilophila wadsworthia* is a recognized opportunistic pathogen and a close relative of *Desulfovibrio* spp. that appears to have lost its ability to reduce sulfate, but which is also able to efficiently use formate and hydrogen as energy sources for growth. It lost the ability to use sulfate replacing it by taurine as a source of sulfite, which may represent an adaptation to the human organism. Recently, the genome of *B. wadsworthia* was sequenced (http://www.broadinstitute.org/annotation/genome/Bilophila_group/MultiHome.html), which will contribute to learning more about this organism's energy metabolism and how the use of different growth substrates relates with its pathogenicity.

Chapter 8

Supplementary information

Supplementary table 8.1. Locus tags for periplasmic FDH genes in SRO analyzed genomes.

		Soluble		Membrane associated	
	Locus	**FdhAB**	**FdhABC3**	**FdhABC**	**FdhABD**
Archaea					
Archaeoglobus fulgidus	AF				
Archaeoglobus profundus	Arcpr_			0674	
Caldivirga maquilingensis	Cmaq_				
Deltaproteobacteria					
Desulfovibrionacae					
Desulfovibrio aespoeensis	DaesDRAFT_	2455; 2841			
Desulfovibrio alaskensis G20	Dde_	0813; 0717; 3513			
Desulfovibrio desulfuricans ATCC 27774	Ddes_		0555		0827
Desulfovibrio magneticus RS-1	DMR_	37460; 24450; 15750			
Desulfovibrio piger	DESPIG_		00975		
Desulfovibrio salexigens	Desal_	2659	1924		
Desulfovibrio sp. FW1012B	DFW101DRAFT_	0146; 0343			
Desulfovibrio vulgaris Hildenborough	DVU	0587	2812		2482
Desulfomicrobiacae					
Desulfomicrobium baculatum	Dbac_	0971; 1570			
Desulfobacteraceae					
Desulfatibacillum alkenivorans	Dalk_				1854
Desulfobacterium autotrophicum HRM2	HRM2_	03430; 40980	47140		
Desulfococcus oleovorans Hxd3	Dole_	1334			
Desulfohalobiacae					
Desulfohalobium retbaense DSM 5692	Dret_	0226			
Desulfonatronospira thiodismutans ASO3-1	DthioDRAFT_	1315; 1367			
Desulfobulbaceae					
Desulfotalea psychrophila	DP			1769; 2986	
Desulfurivibrio alkaliphilus	DaAHT2_			1384	
Syntrophobacteraceae					
Syntrophobacter fumaroxidans MPOB	Sfum_	3510; 1274; 0036			
Clostridia					
Peptococcaceae					
Desulfotomaculum acetoxidans DSM 771	Dtox_				
Desulfotomaculum reducens	Dred_				1114
Candidatus Desulforudis audaxviator MP104C	Daud_				1580
Thermoanaerobacterales					
Ammonifex degensii KC4	Adeg_		0283		
Nitrospirae					
Thermodesulfovibrio yellowstonii	THEYE_	A0891			

Supplementary table 8.2. Locus tags for periplasmic hydrogenases genes in SRO analyzed genomes.

		Periplasmic [NiFe]				Periplasmic [FeFe]	
		Soluble		Memb		Soluble	Memb
	Locus	HynAB	HysAB	HynABC	HynABC3	HydAB	[FeFe]memb
Archaea							
Archaeoglobus fulgidus	AF			1380			
Archaeoglobus profundus	Arcpr_			1005			
Caldivirga maquilingensis	Cmaq_						
Deltaproteobacteria							
Desulfovibrionacae							
Desulfovibrio aespoeensis	DaesDRAFT_	1307				3275	
Desulfovibrio alaskensis G20	Dde_	2138	2135		3756	2281	
Desulfovibrio desulfuricans ATCC 27774	Ddes_	1038		0836		1503	
Desulfovibrio magneticus RS-1	DMR_	15600/15610				12960	
Desulfovibrio piger	DESPIG_	02534	00296				
Desulfovibrio salexigens	Desal_	1916	2049			1332	
Desulfovibrio sp. FW1012B	DFW101DRAFT_	0356				3123	
Desulfovibrio vulgaris Hildenborough	DVU	1922	1918		2526	1769	
Desulfomicrobiacae							
Desulfomicrobium baculatum	Dbac_	0766	1969				
Desulfobacteraceae							
Desulfatibacillum alkenivorans	Dalk_	2276					
Desulfobacterium autotrophicum HRM2	HRM2_	22360	11680				
Desulfococcus oleovorans Hxd3	Dole_						
Desulfohalobiacae							
Desulfohalobium retbaense DSM 5692	Dret_	0266					
Desulfonatronospira thiodismutans ASO3-1	DthioDRAFT_						
Desulfobulbaceae							
Desulfotalea psychrophila	DP	0159		0575			
Desulfurivibrio alkaliphilus	DaAHT2_	0520		0895			
Syntrophobacteraceae							
Syntrophobacter fumaroxidans MPOB	Sfum_	2952				0848	
Clostridia							
Peptococcaceae							
Desulfotomaculum acetoxidans DSM 771	Dtox_						0172
Desulfotomaculum reducens	Dred_						0463
Candidatus Desulforudis audaxviator MP104C	Daud_						0157
Thermoanaerobacterales							
Ammonifex degensii KC4	Adeg_		1787				0290
Nitrospirae							
Thermodesulfovibrio yellowstonii	THEYE_		A1365				

Supplementary table 8.3. Locus tags for selectd cytochromes *c* genes in SRO analyzed genomes.

	Locus	c3	c554-like	split-Soret	NrfHA	c553	Cyt oxidase
Archaea							
Archaeoglobus fulgidus	AF						
Archaeoglobus profundus	Arcpr_						
Caldivirga maquilingensis	Cmaq_						
Deltaproteobacteria							
Desulfovibrionacae							
Desulfovibrio aespoeensis	DaesDRAFT_	1104; 2527	1151		2320/2321		
Desulfovibrio alaskensis G20	Dde_	3182	2858	3211		1821	1823
Desulfovibrio desulfuricans ATCC 27774	Ddes_	2013		2150	0081/0082	1457	
Desulfovibrio magneticus RS-1	DMR_	02560; 21540	02420; 29160; 35840		06520/06510	06240	14880
Desulfovibrio piger	DESPIG_	02928		02367	01830/01831		
Desulfovibrio salexigens	Desal_	0756; 1385; 2447	0603		0750/0751		
Desulfovibrio sp. FW1012B	DFW101DRAFT_	0515; 1530	2927; 2632		1200/1201	2843	0413
Desulfovibrio vulgaris Hildenborough	DVU	3171	0702; 0922		0625/0624	3041; 1817	1815
Desulfomicrobiacae							
Desulfomicrobium baculatum	Dbac_	1057; 3004	0249		2097/2096	1543	1541
Desulfobacteraceae							
Desulfatibacillum alkenivorans	Dalk_	2817; 4187	4484				
Desulfobacterium autotrophicum HRM2	HRM2_	42360	30020				15110
Desulfococcus oleovorans Hxd3	Dole_	3170; 0267		3017			
Desulfohalobiacae							
Desulfohalobium retbaense DSM 5692	Dret_	0413; 1266; 1800; 2420		2360			
Desulfonatronospira thiodismutans ASO3-1	DthioDRAFT_	1352; 2183; 2529	0490	2219/2220	0534/0535		
Desulfobulbaceae							
Desulfotalea psychrophila	DP				0344/0343		
Desulfurivibrio alkaliphilus	DaAHT2_		2272; 0691; 2110; 2097	0012	0065		0175
Syntrophobacteraceae							
Syntrophobacter fumaroxidans MPOB	Sfum_	4047	1622		2398/2399		
Clostridia							
Peptococcaceae							
Desulfotomaculum acetoxidans DSM 771	Dtox_						
Desulfotomaculum reducens	Dred_				0701/0700		
Candidatus Desulforudis audaxviator MP104C	Daud_						
Thermoanaerobacterales							
Ammonifex degensii KC4	Adeg_				1892/1893		
Nitrospirae							
Thermodesulfovibrio yellowstonii	THEYE_	A1056	A1340	A1336	A0193/A0192		